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UNIVERSITY OF ALBERTA

CYTOKINE REGULATION OF MACROPHAGE PROLIFERATION

©

BY

DONALD ROBERT BRANCH

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY.**

IN

MEDICAL SCIENCES (IMMUNOLOGY)

EDMONTON, ALBERTA

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Dr. Bruce Beutler (External Reviewer)
Dr. Tim Mosmann for Dr. Bruce Beutler

September 23, 1992

This thesis is dedicated to my late grandfather, Eugene Defilippis, who always referred to me as "the Professor," and to my second child, yet unborn, who will undoubtedly call me other names.

ABSTRACT

The major objective of this thesis research was to assess the growth factor requirements for the proliferation of mouse macrophages. Previous studies have shown macrophage progenitors to require at least two factors for growth, CSF-1 plus GM-CSF or IL-3. Whether more differentiated growth-competent macrophages require multiple growth factor signals and whether CSF-1 is the major mitogenic agent for these cells has not been adequately examined.

Using well-defined homogeneous populations of primary bone marrow-derived macrophages (BMM), only the cytokine TNFα, or the related molecule TNFβ, which alone had not effect on BMM proliferation, was shown to synergistically cooperate with maximally stimulatory levels of CSF-1. Similar results with a closely matched cell line indicated that the synergistic effect of TNFα is not mediated through contaminating non-macrophage accessory cells. Also, CSF-1 stimulation resulted in increased levels of TNFα mRNA and induced BMM to produce low levels of TNFα. Moreover, different stimuli appear to differentially regulate the nature, extent and location of TNFα proteins. Thus, LPS induces a maximal amount of TNFα (propeptides of 25 kD, 29 kD and 33 kD) with most of the bioactivity found released into the culture medium. In contrast, CSF-1 induces 2 propeptide isoforms (24 kD, 27 kD) and GM-CSF can induce 3 isoforms (20 kD, 24 kD, 27 kD), with bioactivity mainly found associated with the cell. The observation that CSF-1 stimulates TNFα production and that the two cytokines cooperatively stimulate macrophage proliferation, suggests an autocrine function. Indeed, the growth of CSF-1-stimulated BMM was consistently inhibited by neutralizing antiserum to TNF.

The results of this thesis research provide the first evidence that proliferation of relatively homogeneous mature, growth-competent mouse macrophages requires at least two signals for full mitogenic stimulation. CSF-1 appears to be the major mitogenic agent yet examined for these cells, however, CSF-1 does not send an optimal mitogenic signal but may require either exogenous (paracrine) or endogenous (autocrine/juxtacrine/intracrine) TNFα. A possible second signal was shown to be provided by endogenously produced TNFα, thus, the results indicate the first evidence for autocrine growth

stimulation in normal non-lymphoid hematopoietic cells. Although no evidence was obtained that demonstrated that CSF-1 cannot act alone, the results argue that CSF-1 alone does not send an optimal mitogenic signal to mouse macrophages.

ACKNOWLEDGEMENTS

I am very grateful to the following individuals for providing cell lines, cytokines, or other reagents, used in this Thesis work: Dr. T. Beck, Dr. M. Crainie, Dr. J. Elliot, Dr. N. Gough, Dr. D.R. Green, Dr. A. Janowska-Wieczorek, Dr. D. Kunimoto, Dr. P. Lansdorp, Dr. K-C. Lee, Dr. P. Lomedico, Dr. R. Murphy, Dr. V. Paetkau, Dr. A. Rabinovitch, Dr. P. Ralph, Dr. M. Sadelain, Dr. A.R.E. Shaw, Dr. Y. Shi, Dr. E.R. Stanley, Dr. C. Stock, and Dr. R. Tushinski. I also acknowledge the assistance and helpful discussions of Dr. P. Barker, Dr. J. Chiavetta, Dr. L. Chrisey, Dr. A. Janowska-Wieczorek, Dr. D.R. Green, Dr. J. Madrenas, Dr. L.E. McGann, V. Rammassar, Dr. M. Sadelain, Dr. B.M. Sahai, Dr. A.R. Turner, Dr. C. Upton, J. Urmson, Dr. M. Vorelia, and Dr. T.G. Wegmann.

The superb technical assistance and friendship of all individuals in the laboratory, but especially Naisan Garraway, Karen Hobbs, Lynn Linklater, Valerie Narayana, Angie Shah, Randy Stadt, Anne Smith and Bonnie Winkler-Lowen is also greatly appreciated. I also thank Debbie Neufeld for help in the preparation of this thesis.

I am grateful for the friendship and helpful discussions with all of my fellow graduate students, but particularly Dr. Patrick Kilgannon, Dr. Marian Laderoude, Dr. Hector Mayani, Donna McNeil, Ken Muldrew, Dr. Ed Peters, Dr. Michel Sadelain, Dr. Yufang Shi, Dr. Simon Vassiliadis, and Dr. Michael Vorelia.

I thank Tom Mueller for taking me to lunch the first day in Edmonton, and for his friendship. My sincere gratitude also goes to Dr. Jean-Michel Turc for bringing me to Canada, and to the Canadian Red Cross Society for their support.

I give my thanks and my love to my wife, Cathy, and my daughter, Cara-Lynn, for their patience and understanding over the past seven years.

Finally, I acknowledge my supervisor, Dr. Larry J. Guilbert, for taking a chance with an "old man" by accepting me as his graduate student and being supportive and interactive with me throughout the duration. I hope that a fraction of the approach to science which Larry follows will rub off onto me and that I will benefit from the cautious scientific rigor which he has attempted to pass on to me.

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LIST OF ABBREVIATIONS

^{125}I-CSF-1 - Iodine 125 radiolabelled CSF-1
5/10.14 - CSF-1-dependent v-*myc*-transformed murine macrophage cell line
[^{3}H]TdR or ^{3}H-TdR or [^{3}H] thymidine - tritiated thymidine
AMD - Actinomycin D
ATCC - American Type Culture Collection
BFU-E - Burst-forming unit, erythroid
BFU-Meg - Blast-forming unit megakaryocyte
BMM - Bone marrow-derived macrophages
BSA - Bovine Serum Albumin
C - Celsius
cDNA - Complementary DNA
c-*fms* - Cellular oncogene encoding the receptor to CSF-1
CFU - Colony-forming unit
CFU-b - Colony-forming unit, basophil
CFU-Bl - Colony-forming unit, blast cell
CFU-c - Colony-forming unit, culture
CFU-E - Colony-forming unit, erythroid
CFU-Eo - Colony-forming unit, eosinophil
CFU-GEMM - Colony-forming unit granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM - Colony-forming unit granulocyte, macrophage
CFU-LM - Colony-forming unit, lymphoid, myeloid
CFU-m - Colony-forming unit, macrophage
CFU-Mast - Colony-forming unit, mast cell
CFU-Meg/E - Colony-forming unit megakaryocyte, erythroid
CFU-Mix - Colony-forming unit, mixed cellular
CFU-n - Colony-forming unit, neutrophil
CFU-S - Colony-forming unit, spleen

CHO	Chinese hamster ovary
Ci	Curie
CM	Conditioned medium
CO_2	Carbon dioxide
COS	Monkey fibroblasts
cpm	Counts per minute
CRU	Competitive repopulating unit
CSF	Colony-stimulating factor
CSF-1	Colony-stimulating factor-1; also termed M-CSF
DA-1	Non-adherent murine lymphoid cell line
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunoassay
Epo	Erythropoietin; erythroid specific hormone
FBS	Fetal bovine serum
fg	Femtogram
fmol	Femtomole
g	gram
G-CSF	Granulocyte colony-stimulating factor
GM-CFC	Granulocyte-macrophage colony-forming cell
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCl	Hydrochloric acid
HGF	Hematopoietic growth factor
h or hrs	Hours
HPP-CFC	High proliferative potential colony-forming cell
IFN	Interferon
IGF	Insulin growth factor
IgG	Immunoglobulin, gamma
IgM	Immunoglobulin, mu
IL	Interleukin
IMB	IMDM lacking bicarbonate adjusted to pH 7.35 with NaOH

IMDM - Iscove's modified Dulbecco's medium
IU - International units
kb - Kilobase
kD - Kilodalton
Kd - Kinetics of dissociation; binding affinity constant
L - Litre
L cell - Fibroblast cell line
LIF - Leukemia inhibitory factor
LPS - Lipopolysaccharide
LT - Lymphotoxin; also termed TNFβ
M - Molar (moles per litre)
mAb - Monoclonal antibody
M-CSF - Macrophage colony-stimulating factor; also termed CSF-1
mg - Milligram
MGF - Macrophage growth factor
mL - Milliliter
mm - Mllimeter
mole - g/mw
mOsm - Milliosmoles
MTT - Tetrazolium dye: (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide
Multi-CSF - IL-3
MW - Molecular weight
N_2 - Nitrogen
ng - Nanograms
NGF - Nerve Growth Factor
nm - Nanometer
NRS - Normal rabbit serum
OD - Optical density
p - Pico
PBS - Phosphate buffered saline
PDGF - Platelet derived growth factor

pg - Picogram
PGE_2 - Prostaglandin E_2
pH - -log [H^+]
pmol - Picomole
pre-CFC_{multi} - pre-colony-forming cell, multi-lineage
r - Recombinant
RIA - Radioimmunoassay
rpm - Revolutions per minute
RPMI - Roswell Park Memorial Institute
RRA - Radioreceptor assay
S1 - Growth factor-dependent v-*myc*-transformed murine macrophage cell line
SD - Standard deviation
S-oligo - Phosphorothioate oligodeoxynucleotide
TGF - Transforming growth factor
TNF - Tumor necrosis factor
Tris - (Hydroxymethly) Aminomethane
U - Units
μCi - Microcurie
μg - Microgram
μL - Microlitre
vol/vol - Unit of measure
WEHI - Walter and Eliza Hall Institute

CHAPTER I

INTRODUCTION

OVERVIEW

The mature blood cells which effect host defenses against infection and tissue rejection (lymphocytes, granulocytes and macrophages), carry oxygen (erythrocytes) and maintain hemostasis (macrophages and platelets) derive from only a very few specialized cells found in the bone marrow (Golde and Gasson, 1988). These so-called pluripotential stem cells comprise approximately 0.001% to 0.003% of all nucleated bone marrow cells and are capable of both self renewal, i.e., they have the capacity to replicate into identical daughter progeny, and differentiation into committed hematopoietic progenitor cells. These give rise to all lympho-hematopoietic cells of the body (Till and McCulloch, 1961; Wu *et al.,* 1967; Erslev and Gabuzda, 1985; Metcalf, 1984).

The regulation of hematopoiesis may involve both stochastic events as well as deterministic influences which generate the different lympho-hematopoietic cell lineages (Till and McCulloch, 1961; Wu *et al.,* 1967; Curry and Trentin, 1967; Trentin, 1971; see Figure I-1). Soluble humoral factors (or cytokines) comprise a group of regulatory proteins and glycoproteins termed colony stimulating factors (CSFs) and interleukins. These cytokines act on early and late developing cells to modulate their maturation and proliferation through various stages of lineage development (Clark and Kamen, 1987; Whetton and Dexter, 1989).

Which and how cytokines may regulate the survival, growth and differentiation of hematopoietic cells of different lineages has become increasingly important. This is partly because of a relationship of oncogenes and growth factors with the potential for hematopoietic cell transformation. This suggests an important role for cytokine dysregulation in human hematopoietic malignancies (Pierce, 1989). Cytokines also are beginning to be used in the clinic. For example, erythropoietin (Epo) is used for the treatment of anemia and granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used to shorten the time necessary for the reconstitution of transplanted bone marrow.

Most *in vitro* studies of cytokine regulation of hematopoietic cells are complicated by the use of relatively heterogeneous target cell populations, i.e., bone marrow, making it difficult to distinguish direct effects of cytokine action from indirect effects mediated by accessory cells contaminating the target cell population. Moreover, the study of cytokine regulation of hematopoietic cell growth is further complicated by the inevitable involvement of cytokine networks (Moore *et al.*, 1990), i.e., the simultaneous or sequential action of different cytokines which regulate both positive and negative aspects of growth stimulation. In addition, many hematopoietic cells are capable of endogenous production of regulatory cytokines for which these cells have the corresponding cell surface receptors, adding an additional complication to the study of cytokine effects on hematopoietic cells.

To circumvent some of these problems, I have chosen to examine the proliferation of relatively mature mononuclear phagocytes, both primary cells and cell lines, derived from mouse bone marrow. These bone marrow-derived macrophages (BMM) are easily purified and, in mouse, are dependent upon cytokines for their survival and proliferation (Stanley *et al.*, 1983). The use of pure, natural or recombinant cytokines, plus the use of cell lines, in conjunction with primary cell populations, should help to control for indirect effects mediated by crude cytokine preparations or contaminating non-macrophage accessory cells. In addition, the primary cell populations provides physiologic relevance for normal macrophage responses.

This chapter will review aspects of mononuclear phagocyte biology and hematopoietic growth factors and provide rationale for development of the thesis. In addition, it is the purpose of this chapter to acquaint the reader with the concepts of multi-factor growth regulation and autocrine growth regulatory mechanisms as they may relate to this thesis and to the growth and proliferation of mouse macrophages.

MONONUCLEAR PHAGOCYTES

The mononuclear phagocyte system is comprised of the nonlymphoid macrophage cell lineage (Clark and Kamen, 1987; Golub and Green, 1991). This myeloid cell lineage is thought to derive from a single hematopoietic stem cell of bone marrow origin. Under the influence of microenvironmental and hematopoietic growth factors, this stem cell compartment begins differentiation into a series of early, committed multipotential myeloid progenitor cells, the last of which is bipotential and termed a colony forming unit-granulocyte macrophage (CFU-GM) (see Figure I-1). The CFU-GM gives rise to neutrophil (CFU-n) or macrophage (CFU-m) progenitors which in turn continue to proliferate and mature into effector neutrophils and macrophages. Thus, under the influence of macrophage-specific growth factors, CFU-m continue differentiation along a unipotential pathway, within the bone marrow, into an early monocytic cell termed a monoblast. The monoblast further differentiates into a promonocyte and then a monocyte. The monocytes leave the bone marrow, enter the peripheral blood and subsequently are transported to the body's organs and cavities where they develop into cells termed macrophages (van Furth and Cohn, 1978; Bol *et al.*, 1979; Metcalf, 1980). Mature macrophages have different morphological and functional features depending upon which tissue these cells settle in (van Furth, 1985).

The monocyte-macrophage is important in host defense (van Furth, 1985; Paul, 1989) serving as an important mediator for immunological responses. Macrophages mediate the processing and presentation of immunologically relevant antigens to T cells (Paul, 1989; Drysdale *et al.*, 1988). Monocyte-macrophages also are major producers of immunologically important proteins (Nathan, 1987; Durum and Oppenheim, 1989). In addition, monocyte-macrophages serve as important mediators of inflammation (Sakkonen *et al.*, 1990), wound healing and in the removal of aged blood cells (Kay, 1991).

The growth of macrophages *in vitro* is thought to require the presence of a specific hematopoietic growth factor known both as macrophage-colony-stimulating factor (M-CSF; Metcalf, 1986) and colony-stimulating factor-1 (CSF-1; Stanley *et al.*, 1983). In humans, mononuclear phagocyte proliferation does not occur much beyond the promonocyte stage, except, possibly, in the case of injury or inflammation. In contrast, in the mouse system, proliferation of mononuclear phagocytes proceeds throughout the cell lineage, up until the highly differentiated mature tissue-fixed macrophage, although the later developing cells have a lower proliferative potential than earlier cells of the lineage (Stanley *et al.*, 1983; Chen *et al.*, 1987). Thus, proliferation of mature 'young' mouse macrophages (just beyond the monocyte stage) can be studied *in vitro*, and these cells can be obtained in high yield as relatively homogeneous populations (Tushinski *et al.*, 1982).

One of the most fascinating aspects of mononuclear phagoctye biology is the number of biologically important molecules that these cells are capable of producing. Their responses include secretion of more than 100 molecules ranging in molecular mass from 32 (superoxide anion) to 440,000 (fibronectin) and include biologic activities for cellular growth and cellular death (Nathan, 1987).

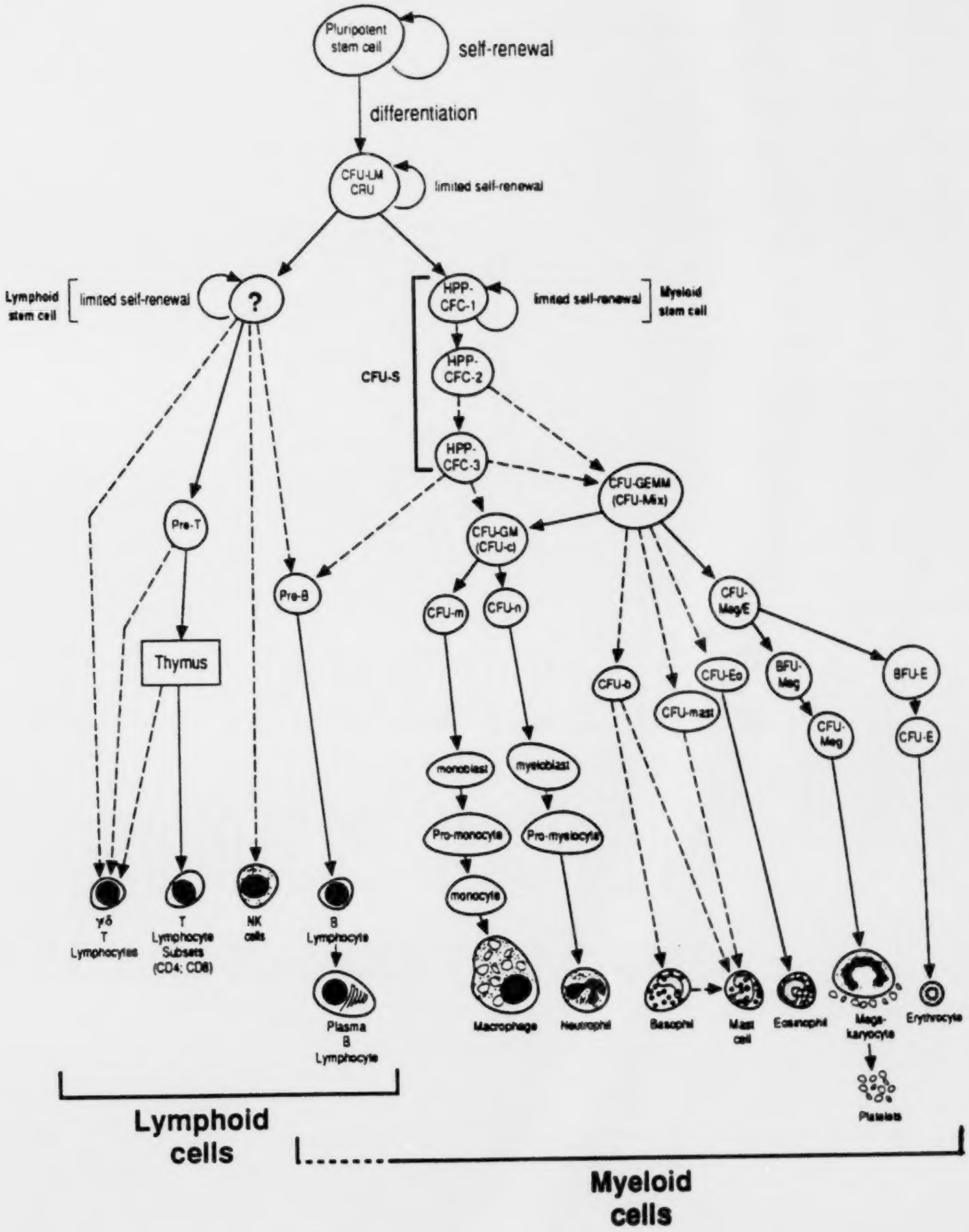
The production from mononuclear phagocytes of molecules which can function as regulators of hematopoietic activity (often, incorrectly, termed monokines) include CSF-1 (Oster *et al.*, 1987), granulocyte-CSF (G-CSF; Metcalf and Nicola, 1985; Piacibello *et al.*, 1985), GM-CSF (Sullivan *et al.*, 1984; Fibbe *et al.*, 1986), interleukin 1 (IL-1; Durum and Oppenheim, 1989), IL-3 (Rich, 1986), IL-6 (Durum and Oppenheim, 1989), Epo (Rich, 1988), tumor necrosis factor-α (TNFα; Warren and Ralph, 1986; Old, 1989; Durum and Oppenheim, 1989) and CSF-1 (Horiguchi *et al.*, 1987; Oster *et al.*, 1987; Rambaldi *et al.*, 1987). Many of these cytokines are produced during an inflammatory response.

Mice of the C3H/HeJ strain have a deletion in chromosome 4 (Rosenstreich *et al.*, 1978) that impairs their response to the bacterial endotoxin lipopolysaccharide (LPS). This mutation renders these mice highly refractory to endotoxin, the LPS component derived from gram-negative bacterial membranes, and specifically, to the lipid A moiety of the LPS molecule (Sultzer, 1968; Watson *et al.*, 1978). Macrophages from these mice have been reported to be markedly less capable than normal macrophages of producing inflammatory-related cytokines when challenged with LPS (Beutler *et al.*, 1986a). These include TNFα (Beutler *et al.*, 1986a), IL-1-α (Rosenstreich *et al.*, 1978), and GM-CSF

Figure I-1

Hierarchy of lymphohematopoiesis

Hierarchy of lymphohematopoiesis. Solid lines indicate probable or known pathways. Broken lines indicate speculated. CFU-LM, colony-forming unit, lymphoid, myeloid; CRU, competitive repopulating unit; HPP-CFC, high proliferative potential colony-forming cell; CFU-S, colony-forming unit, spleen; CFU-GM, colony-forming unit, granulocyte/macrophage; CFU-c, colony-forming unit, culture; CFU-m, colony-forming unit, macrophage; CFU-n, colony-forming unit, neutrophil; CFU-GEMM, colony-forming unit, granulocyte/erythroid/ macrophage/megakaryocyte; CFU-Mix, colony-forming unit, mixed cellular; CFU-Meg/E, colony-forming unit megakaryocyte, erythroid; BFU-Meg, burst-forming unit, megakaryocyte; CFU-Meg, colony-forming unit, megakaryocyte; CFU-mast, colony-forming unit, mast cell (postulated); CFU-b, colony-forming unit, basophil (postulated); CFU-Eo, colony-forming unit, eosinophil, BFU-E, burst-forming unit, erythroid; CFU-E, colony-forming unit, erythroid. Other cell types may precede the CFU-S compartment: these include pre-$\text{CFC}_{\text{multi}}$; pre-colony-forming cell, multi-lineage and CFU-Bl, colony-forming unit, blast cell. There is good evidence for the myeloid stem cell, however, the lymphoid stem cell is postulated. B lymphocytes can share features of both lymphocytes and myeloid cells, particularly, properties of macrophages.


Pluripotent stem cell
self-renewal
differentiation
CFU-LM CRU
limited self-renewal
Lymphoid stem cell
limited self-renewal
?
HPP-CFC-1
limited self-renewal
Myeloid stem cell
CFU-S
HPP-CFC-2
HPP-CFC-3
CFU-GEMM (CFU-Mix)
Pre-T
Pre-B
CFU-GM (CFU-c)
CFU-m
CFU-n
CFU-Meg/E
Thymus
CFU-b
CFU-mast
CFU-Eo
BFU-Meg
BFU-E
CFU-Meg
CFU-E
monoblast
myeloblast
Pro-monocyte
Pro-myelocyte
monocyte
γ-δ T Lymphocytes
T Lymphocyte Subsets (CD4; CD8)
NK cells
B Lymphocyte
Plasma B Lymphocyte
Macrophage
Neutrophil
Basophil
Mast cell
Eosinophil
Mega-karyocyte
Erythrocyte
Platelets
Lymphoid cells
Myeloid cells

(Apte and Pluznik, 1976). Furthermore, LPS has been shown to down-regulate CSF-1 receptor levels on normal LPS-responsive C3H/HeN BMM but not C3H/HeJ BMM (Guilbert and Stanley, 1984). This suggests that BMM from LPS-hyporesponsive mice (C3H/HeJ) may respond differently to exogenous mediators of proliferation than will BMM from normal LPS-responsive animals such as C3H/HeN mice. However, the ability of macrophages from C3H/HeJ animals to respond to or produce cytokines has not been thoroughly examined.

Relevance to thesis

Mononuclear phagocytes can be obtained in high yield and purity from mouse bone marrow. These BMM are growth factor-dependent and may be a good model system for the examination of both positive and negative regulatory effects of soluble mediators on the *in vitro* proliferation of these cells. Also, the potential for hypoproduction of certain cytokines by C3H/HeJ BMM is exploited and studied in this thesis. Thus, in addition to normal, LPS-responsive C3H/HeN mouse macrophages, I decided to use C3H/HeJ macrophages, both normal primary BMM and a cell line, in order to circumvent potential growth modulatory effects on proliferation due to LPS contaminants (Guilbert and Stanley, 1984), present in virtually all tissue culture systems. In addition, I thought that use of C3H/HeJ cells may allow for detection of cooperative cytokine effects which may go undetected using normal cells, due to a potential masking effect of endogenously produced LPS-inducible growth-modulatory cytokines.

HEMATOPOIETIC CYTOKINES

The development of culture techniques using semi-solid medium was the major breakthrough which allowed for the *in vitro* study of the growth of hematopoietic cells. The initial culture methods (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966) allowed for the identification and study of colonies of mature macrophages and granulocytes when hematopoietic cells of bone marrow origin were immobilized in a soft gel, such as agar or methylcellulose, and provided with "growth factors" contained in various crude extracts. These colony forming assays resulted in the identification of colony stimulating factors (CSFs) for macrophage (CSF-1), granulocyte (G-CSF) and granulocyte-macrophage (GM-CSF) production. Further advances in these culture techniques allowed for conditions which promoted growth of cells of other hematopoietic lineages (Iscove and Sieber, 1975; Metcalf, 1977).

This early work led to the concept that cellular survival, growth and differentiation is strongly influenced (and, may in fact, be controlled) by humoral factors. Furthermore, these CSFs can be lineage nonspecific (pluripoietins) or lineage restricted (unipoietins).

Since the early work on hematopoietic growth factors, many growth regulatory molecules have been identified, characterized, cloned, expressed and made available to investigators. These molecules include CSFs, the interleukins, tumor necrosis factors, transforming growth factors, and many other molecules, some yet to be discovered, which are produced by cells and regulate cellular growth, proliferation and survival. These molecules are now collectively termed cytokines.

The availability of cytokines in a pure recombinant form has led to the observations *in vitro* that each of these hematopoietic growth factors demonstrates pleotropic effects. That is, a given factor can act on a variety of cell types and has the potential to induce different functional activities depending upon the cellular target. This implies that the specific receptors for these growth factors are present on a variety of cells and this, in general, is the case. Indeed, specific high-affinity receptors, unique for each hematopoietic growth factor, have been identified on a variety of cell types.

Myeloid growth factors

Although there are many factors involved in blood cell formation, five cytokines have been described as the major growth modulating agents for nonlymphoid cells of the hematopoietic cell lineage. These myeloid colony-stimulating glycopolypeptides are IL-3, GM-CSF, G-CSF, CSF-1 and Epo. Two of these growth factors, IL-3 and GM-CSF, are pluripoietins having growth stimulating activity for progenitors of most myeloid cell types, including granulocytes, macrophages, megakaryoctyes and erythrocytes (Nicola, 1989). The others are lineage restricted, acting on more differentiated cell types; G-CSF stimulates the growth of granulocytes, primarily neutrophils; CSF-1 the growth of monocyte-macrophages; and Epo the growth of erythroid cells. All five factors, both mouse and human, have been isolated, cloned and expressed. This has allowed extensive characterization of their effects, both *in vitro* and *in vivo* (reviewed in Leary *et al.*, 1987; Dexter, 1987; Klingeman and Eaves, 1988; Platzer, 1989; Gillis, 1989; Whetton and Dexter, 1989).

Functional activity requires binding of each factor to a single class of high-affinity receptor and there is no crossreactivity with other receptors, with the exception of GM-CSF and IL-3 which show limited cross-reactivity (Nicola, 1987a; Lopez *et al.*, 1989; Kitamura *et al.*, 1991; see below). Only IL-3, GM-CSF and CSF-1 have been shown to have mitogenic activity for growth factor-dependent mouse macrophages (Guilbert, 1985; Hamilton *et al.*, 1988). As my thesis examines the regulation by cytokines of the proliferation of relatively mature growth-competent mouse macrophages, I will limit my discussion of the myeloid growth factor cytokines to only IL-3, GM-CSF and CSF-1.

IL-3

IL-3 also known as multi-CSF, was first identified by its ability to induce the expression of 20α-steroid dehydrogenase in splenic lymphocytes from nude mice (Ihle *et al.*, 1981). Subsequent to its purification (Ihle *et al.*, 1982), IL-3 was also found to be a growth promoting agent for many different cell types including granulocyte, macrophage, eosinophil, mast cell, megakaryocyte, natural killer (NK), erythroid and multipotent colonies from mouse fetal liver and bone marrow and, possibly, early T and B lineage cells (reviewed in Whetton and Dexter, 1989).

The biology and biochemistry of mouse and human IL-3 has been reviewed elsewhere (Metcalf, 1986; Clark and Kamen, 1987; Platzer, 1989; Gillis, 1989; Whetton and Dexter, 1989). However, certain aspects concerning the biology of IL-3 should be noted. (1) IL-3 appears to be the only cytokine capable of stimulating, on its own, multipotent stem cell self-renewal or development (McNiece *et al.*, 1990). Spooncer and colleagues (Spooncer *et al.*, 1986), showed that IL-3 can promote the development of early stem cells termed colony forming unit-spleen (CFU-S; Till and McCulloch, 1961; See Figure I-1). Indeed, the addition of IL-3 to bone marrow cultures stimulates both the survival and DNA synthesis of the CFU-S (Spivak *et al.*, 1985). Additional reports have confirmed a role for IL-3 in early hematopoietic stem cell development (Ody *et al.*, 1990; Iscove and Yan, 1990). However, this property of IL-3 may require participation of a cofactor such as CSF-1 (McNiece *et al.*, 1990) or IL-1 (Iscove and Yan, 1990). (2) Unlike most cytokines, IL-3 appears to be unusual in that its normal production appears limited to only one cell type. The main source of IL-3 from normal cells is the activated T-cell. However, it has been reported that keratinocytes may also be a source of IL-3 (Luger *et al.*, 1985), and IL-3 has also been reported to be produced by monocyte-macrophages (Rich, 1986), although this finding has not been confirmed (Sieff *et al.*, 1988). (3) Although IL-3 is mitogenic for mouse BMM (Hamilton *et al.*, 1988), the degree of responsiveness is far less than for either CSF-1 or GM-CSF. Nonetheless, it can give rise to macrophage colonies from semisolid agar cultures of bone marrow cells (Metcalf, 1984), stimulate Na^+, K^+ -ATPase activity (Vairo and Hamilton, 1988), and down-modulate, slightly, the binding of CSF-1 to its receptor (Walker *et al.*, 1985). Thus, mouse macrophages retain some responsiveness to IL-3 even after differentiation (Crapper *et al.*, 1985).

IL-3 binds to its receptor with high and low affinities and induces tyrosine phosphorylation. However, the cloned mouse IL-3 receptor (Itoh *et al.*, 1990), when expressed in either COS monkey fibroblasts or on L cell stable transfectants, bound IL-3 with only low affinity (18 nM). Furthermore, no consensus sequence for a tyrosine kinase was present in the cytoplasmic domain. This indicates that the IL-3 binding protein and the tyrosine kinase activity are distinct from each other. The IL-3 receptor belongs to a distinct family of receptors, termed the hematopoietin receptor-gene family (Gillis, 1991), which includes the cytokine receptors for IL-2 (β-chain), IL-4, IL-5, IL-6, IL-7, Epo, G-CSF, LIF and GM-CSF (see below). These receptors all share two conserved motifs within their external domains, including a characteristic serine-tryptophan-

X-serine-tryptophan (WSXWS, where X is any amino acid) motif just proximal to the membrane spanning region of the receptor. Furthermore, members of the hematopoietin receptor-gene family appear to require some association with an additional molecule for full biologic activity. Thus, this family of cytokine binding proteins (receptors) do not, in and of themselves, appear able to provide a "complete" mitogenic signal to the cell (Itoh *et al.,* 1990).

Human IL-3 receptor was shown to consist of two distinct proteins of 70 kD (α subunit) and 120-135 kD (β subunit) by chemical cross-linking (Kuwaki *et al.,* 1989). It has now been shown that the higher molecular weight subunit, termed the β subunit, is a common binding structure for both IL-3 and GM-CSF (Budel *et al.,* 1990; Kitamura *et al.,* 1991a). Recently, the common β subunit for IL-3 and GM-CSF receptor has been cloned and expressed (Kitamura *et al.,* 1991b). This system is unique because the same β subunit can bind two distinct ligands having no significant sequence homology with each other, but only when it forms high affinity receptors with ligand-specific IL-3 or GM-CSF α subunits.

Although much *in vitro* work has been done with IL-3, a physiological role for this molecule has not been firmly established. Macrophages treated *in vitro* with IL-3 can produce G-CSF (Metcalf and Nicola, 1985) and this may have some physiological relevance. However, IL-3 cannot be detected in serum, even after using strong T cell activating agents (Dexter, 1989). Nonetheless, in the mouse, IL-3 may function as a short-range paracrine survival and differentiation factor, primarily for very early hematopoietic cells and cells of the erythroid lineage. However, in man, there is, as yet, little evidence to suggest any physiological role for IL-3.

GM-CSF

GM-CSF is a pluripoietin which is similar to IL-3 in its target cell range. The notable difference is that GM-CSF appears to have no effect on primitive hematopoietic stem cells. Many cell types produce GM-CSF under appropriate conditions, including T lymphocytes, macrophages, fibroblasts, endothelial cells, mesangial cells, keratinocytes, placental cells and brain astrocytes (Chodakewitz *et al.,* 1988; Whetton and Dexter, 1989; Budde *et al.,* 1989; Wegmann *et al.,* 1989;

Malipiero *et al.*, 1990). There are indications that GM-CSF may be important as a cellular regulatory molecule in the bone marrow stroma or possibly play an important physiological role in the inflammatory response. Since GM-CSF only becomes detectable in serum during infection, a possible physiologic role for GM-CSF may also involve control of bacterial and parasitic infections. In support of this idea, GM-CSF has been shown to increase superoxide anion production from neutrophils (Lindemann *et al.*, 1988) and cytotoxic activity of macrophages (Weiser *et al.*, 1987; Belosevic *et al.*, 1988). In addition to direct effects of GM-CSF on monocyte-macrophages, indirect effects have also been reported. For example, the observation that GM-CSF-treated monocytes or macrophages produce inflammatory mediators such as IL-1 (Arnaout *et al.*, 1986), tumor necrosis factor (Cannistra *et al.*, 1987) or CSF-1 (Horiguchi *et al.*, 1987), support a physiological role for GM-CSF in the inflammatory response.

There are three types of GM-CSF receptors: One having low-affinity binding and two distinct high-affinity binding receptors (Gearing *et al.*, 1989). One type of high-affinity binding GM-CSF receptor binds only GM-CSF and is the only type found on human neutrophils. The other high-affinity binding GM-CSF receptor binds both GM-CSF and IL-3 with equal affinity and represents 80% of the GM-CSF receptors on eosinophils (Lopez *et al.*, 1989). As discussed above for the IL-3 receptor, it has been shown that the GM-CSF is a member of the hematopoietin receptor-gene family. Indeed, the GM-CSF receptor consists of two subunits, one of approximately 120 kD that is termed the β subunit (Kitamura *et al.*, 1991a), which is identical to the β subunit of the IL-3 receptor (Kitamura *et al.*, 1991b). The other subunit, the α subunit of GM-CSF receptor is distinct from the α subunit of IL-3 receptor. There are many instances where IL-3 and GM-CSF induce similar signals and show similar biological activities (Metcalf, 1986; Clark and Kamen, 1987). Thus, the elucidation of the β subunit helps to explain these earlier observations.

GM-CSF is capable of inducing the proliferation of mouse macrophages on its own (Guilbert, 1985; Hamilton *et al.*, 1988; Chen *et al.*, 1988). However, under similar culture conditions, GM-CSF is not usually as good a mitogenic agent as CSF-1. The reasons for this are unknown but may relate to higher numbers of CSF-1 receptors as compared to GM-CSF receptors, or to differences in the mitogenic signals. It may be that CSF-1 sends a complete mitogenic signal while GM-CSF sends an incomplete signal. Perhaps the best evidence for GM-CSF acting to send a complete mitogenic signal for the proliferation of mouse macrophage comes from the work with mice made transgenic for the constitutive expression of GM-CSF (Metcalf, 1990a, 1990b). These mice were found to have abnormally high numbers of macrophages which expressed normal or increased GM-CSF receptor levels, suggesting that the increased numbers of macrophages was directly related to stimulation by GM-CSF.

In addition to its direct mitogenic effect, GM-CSF also causes a marked down-modulation of the binding of CSF-1 to its receptor (Walker *et al.*, 1985; Nicola, 1987a; Nicola, 1987b). Indeed, it has been suggested that GM-CSF is a dominant negative regulator of the CSF-1 receptor (Gliniak and Rohrschneider, 1990), that GM-CSF can suppress development along the monocyte/macrophage lineage. This idea has received recent support where a selective reduction in the numbers of macrophage colonies was observed when GM-CSF was combined with CSF-1 (Metcalf and Nicola, 1992). Thus, two positive growth factors (CSF-1 and GM-CSF) may result in a negative effect on growth when mixed together.

CSF-1

CSF-1 also known as M-CSF, is a homodimeric sialoglycoprotein that stimulates the survival, proliferation and differentiation of mononuclear phagocytes (Tushinski *et al.*, 1982; Stanley *et al.*, 1983) and may also play a role in placental development (Pollard *et al.*, 1987), and in the control of immune function in the glomerulus of the kidney (Mori *et al.*, 1990). Investigators have also shown that CSF-1 may be important for early CFU-S proliferation (McNiece *et al.*, 1990). High proliferative potential-colony forming cells (HPP-CFC), which are thought to represent early primitive cells within the CFU-S hematopoietic cell compartment (see Figure I-1), require CSF-1 plus a synergy factor, either IL-1 or IL-3, for growth. Also, the putative mouse Thy-1^{lo}, Sca-1+, stem cell (Spangrude *et al.*, 1988), when separated on a single cell basis and exposed to multiple factors, gives rise to giant macrophage colonies with the phenotype of HPP-CFC (McNiece *et al.*, 1990).

CSF-1 is produced by a variety of normal cells including monocyte-macrophages, fibroblasts, placental cells, mesangial cells, keratinocytes, and activated T and B cells (Metcalf, 1985; Rambaldi *et al.*, 1987; Reisbach *et al.*, 1989; Arceci *et al.*, 1989; Chodakewitz *et al.*, 1990; Mori *et al.*, 1990; Daiter *et al.*, 1992). CSF-1 is also produced by malignant cells such as ovarian epithelial cancer cells (Kacinski *et al.*, 1989). Human CSF-1 has a normal concentration range in blood of between 350 - 700 U/mL (about 100 - 200 pM). Serum CSF-1 levels are elevated in patients having certain malignancies (Kacinski *et al.*, 1990; Janowska-Wieczorek *et al.*, 1991) and remain high only transiently following aggressive cytotoxic treatment or injection of bacterial endotoxin (Dexter, 1989). Very high levels of serum CSF-1 are detectable throughout mouse or human pregnancy (Bartocci *et al.*, 1986).

A variety of cytokines can stimulate CSF-1 production including IFNγ (Horiguchi *et al.*, 1986), GM-CSF (Horiguchi *et al.*, 1987) and TNFα (Oster *et al.*, 1987) suggesting that the release of CSF-1 from mononuclear phagocytes may enhance their local proliferation (or function) during an inflammatory response or at sites of tissue injury.

There is only one gene for CSF-1 (Kawasaki *et al.*, 1985), however, a number of CSF-1-specific mRNAs have been detected in both human and mouse cells, ranging in size from 1.6 kb to 4.5 kb (Ladner *et al.*, 1987; Sherr, 1990a). These data indicate that the different RNA species result from differential processing of the primary CSF-1 transcript. These encode at least three different CSF-1 polypeptides that are processed to related but different forms of mature CSF-1, all of which retain full biologic activity. All of the various forms of CSF-1 are secreted with the notable exception of the protein encoded by the 1.6 kb transcript (Sherr, 1990a). The product of this transcript is a transmembrane polypeptide precursor which lacks recognition sites for the intracellular protease which normally cleaves the CSF-1 precursor to yield a soluble secreted form of CSF-1. Thus, the 1.6 kb transcript generates a plasma membrane-bound form of CSF-1 which retains full biologic activity (Rettenmier *et al.*, 1988). Although the 1.6 kb transcript has not been found in many CSF-1-producing cells, its identification suggests that CSF-1 can exist in both secreted and cell-associated forms, the latter of which implies a role for CSF-1 in cell-cell interactions by a juxtacrine mechanism. Recently, it has been shown that the predominant form of secreted CSF-1 is a proteoglycan (Price *et al.*, 1992).

The actions of CSF-1 are mediated through its binding to specific cell surface receptors encoded by the c-*fms* proto-oncogene (Sherr *et al.*, 1985). The CSF-1 receptor (CSF-1R), unlike the receptors for IL-3, GM-CSF, and Epo, exhibits an integral ligand-dependent protein-tyrosine kinase activity (Sherr *et al.*, 1985; Sherr, 1990b). In addition, the CSF-1R has been found on cell types not of lymphohematopoietic origin, including trophoblasts (Daiter *et al.*, 1992) and normal ovarian epithelium (Kacinski *et al.*, 1989), as well as on a subset of B cells (Cumano *et al.*, 1992). Thus, the CSF-1R is not, as previously thought, an exclusive marker for hematopoietic mononuclear phagocytes.

The CSF-1R is an integral transmembrane glycoprotein having an extracellular domain showing homology with the immunoglobulin gene superfamily, a single hydrophobic membrane-spanning segment, and an intracellular domain which includes all the sequences necessary for tyrosine kinase activity (Downing *et al.*, 1989; Sherr, 1990a; Sherr, 1990b). Binding of CSF-1 activates the receptor kinase, leading to autophophorylation of receptor subunits and the concomitant phosphorylation of a series of cellular proteins on tyrosine residues (Sherr, 1990c) and, in particular, phosphoinositol-3 (PI-3) kinase. The diverse effects of CSF-1 on mononuclear phagocyte survival, proliferation, differentiation and macrophage effector function appear to reflect the ability of CSF-1R to simultaneously modulate the activities of a series of intracellular proteins that function in relaying biochemical signals.

The structure of the CSF-1R is closely related to the A and B isoforms of PDGF receptor, the c-*kit* proto-oncogene product and the receptor for fibroblast growth factor (Sherr, 1990b). The gene for one isoform of the PDGF receptor is located in tandem on chromosome 5 with the CSF-1R gene (Roberts *et al.*, 1988).

CSF-1R can be down modulated by at least three different mechanisms: (1) by direct ligand binding resulting in rapid internalization of CSF-1/CSF-1R complexes (Sherr, 1990b; see Appendix 1k); (2) by release of a specific protease which cleaves the extracellular domain of CSF-1R; this mechanism has been referred to as "transmodulation" (Guilbert *et al.*, 1983; Downing *et al.*, 1989; Sherr, 1990b) and appears to require protein kinase C activation; and (3) a mechanism whereby various cytokines, particularly TNF, cause a transient down-modulation of CSF-1R with receptor levels returning to normal within 4 to 24 hours (Guilbert and Stanley, 1984; Walker *et al.,* 1985; Branch *et al.,* 1989; Shieh *et al.,* 1989). The mechanism of this "trans-acting" effect of cytokines on CSF-1R has not been studied.

There has been considerable speculation regarding the significance of down-modulation of CSF-1R (Nicola, 1987b). However, with so many different agents capable of modulating the CSF-1R (see Appendix 1k), it makes the significance of this effect unclear. In specific instances, down-regulation of CSF-1R may serve, perhaps, to modulate autocrine growth or survival mechanisms (Sherr, 1990b).

Mouse macrophage proliferation is claimed to depend solely on CSF-1 (Tushinski and Stanley, 1985; Matsushime *et al.,* 1991), which is required throughout the G1 phase of the cell cycle to ensure both cell survival and S phase commitment. The best evidence that CSF-1 is the major mitogenic agent for the survival, production and proliferation of mouse macrophages comes from studies on the osteopetrotic (op) mouse. Mice that are mutant at the op locus have a severe deficiency of mononuclear phagocytes, apparently due to an inactivating mutation in the CSF-1 gene (Wiktor-Jedrzejczak *et al.*, 1991). Defects in macrophage numbers are corrected in these animals after administration of CSF-1.

Recently, stimulation of the BAC1.2F5A macrophage cell line by CSF-1 resulted in the isolation of two novel cyclin-like proteins which function during the G1 phase of the cell cycle and may regulate S phase commitment (Matsushime *et al.,* 1991). CSF-1 deprivation during G1 leads to rapid degradation of a 36 kD cyclin and correlates with failure to initiate DNA synthesis. Thus, CSF-1 may regulate the proliferation of mouse macrophages through its regulatory effect on specific, unique cyclin-like genes.

Relevance to thesis

CSF-1, GM-CSF and IL-3 all stimulate the *in vitro* proliferation of mouse BMM (Guilbert, 1985, Branch and Guilbert, 1986; Hamilton *et al.,* 1988; Chen *et* *al.*, 1988). In addition, these three cytokines can down-regulate the CSF-1 receptor on mononuclear phagocytes (Walker *et al.*, 1985). The ranking of these cytokines in order of mitogenic effect is CSF-1>GM-CSF>IL-3 (Guilbert, 1985; Branch and Guilbert, 1986; Hamilton *et al.*, 1988). However, this hierarchy of response may reflect the fact that in these studies, the macrophages were derived under the influence of CSF-1. The order of response may be different if the macrophages are derived in the presence of GM-CSF, IL-3 or combinations of these cytokines. Indeed, the mitogenic response of CSF-1-derived BMM correlates with the receptor number. Likewise the proliferation of macrophages in mice made transgenic for GM-CSF production appears to correlate with the GM-CSF receptor levels (Metcalf, 1990b). Thus, it has not yet been established whether all macrophages resemble CSF-1-derived BMM. Therefore, whether CSF-1 is the major mitogenic agent for the proliferation of other macrophages is unknown. Furthermore, many cytokines have not been examined for their potential growth modulatory effect on the proliferation of mouse mononuclear phagocytes. Also, whether the CSF-1 receptor alone sends a complete mitogenic signal or requires costimulatory signals for its full mitogenic response are questions that need examination.

CYTOKINES PRODUCED BY MONONUCLEAR PHAGOCYTES

Cytokines which are produced by cells of the mononuclear phagocyte cell lineage have been termed "monokines". However, this terminology is misleading in that it implies some uniqueness to the cytokine i.e., a cytokine made only by and/or acting on macrophages. In fact, most of the cytokines produced by mononuclear phagocytes can be produced by other cells of hematopoietic or nonhematopoietic origin and these cytokines have pleotropic effects. Thus, the term "monokine" is incorrect and should no longer be used for these cytokines.

A number of cytokines produced by macrophages are made during an inflammatory response, including GM-CSF, TNFα and IL-1. Messenger RNA of these inflammatory cytokines have a common motif present within their 3' untranslated region. This highly conserved UUAUUUAU octanucleotide sequence has been shown to be capable of directing mRNA degradation (Shaw and Kamen, 1986; Wilson and Treisman, 1988). This degradative pathway appears to require a specific ribonuclease (Beutler and Cerami, 1988). In addition to its contributory role in the instability of these mRNA, it appears that these UUAUUUAU sequences need to be cleaved in order to initiate protein translation (Kruys *et al.*, 1989). These sequences, which play a major role in the dynamics of expression of inflammatory cytokines, are highly regulated by bacterial endotoxin (Beutler *et al.*, 1986). One of these molecules, TNFα, is a focus of my thesis research and its biology and physiology are discussed below.

Tumor necrosis factor-alpha (TNFα)

General properties

TNFα was initially detected in the serum of mice, primed by infection with *Bacillus Calmette-Guerin* and subsequently injected with endotoxin, as an activity that induces hemorrhage and necrosis of transplanted mouse and human tumors (Carswell *et al.*, 1975). TNFα also has direct cytotoxic and cytostatic effects on a variety of human cancer cells *in vitro* (Old, 1988; Ruddle, 1987; Beutler and Cerami, 1989). It is now recognized that TNFα is a molecule with pleotropic activities that shares many biological activities with IL-1 (Le and Vilcek, 1987). TNFα serves as a mediator of inflammation and is an inducer of shock, as well as playing a critical role in immune modulation, tissue remodeling, and a wasting syndrome known as cachexia (Beutler *et al.*, 1985a; Beutler *et al.*, 1985b; Beutler *et al.*, 1985c; Brenner, 1988; Sherry and Cerami, 1988; Beutler and Cerami, 1988; Tracey *et al.*, 1989; Beutler and Cerami, 1989; Beutler, 1990a; Beutler, 1990b; Warren, 1990; Jacob *et al.*, 1991; Fiers, 1991). Although mouse TNFα and human TNFα, like G-CSF, cross-react, there is some relative species specificity with TNFα (Smith *et al.*, 1986). For example, in cytotoxicity assays, human TNFα is more cytotoxic than mouse TNFα for human HeLa cells while against mouse L929 cells, mouse TNFα is more cytotoxic than human TNFα (Kramer and Carver, 1986).

TNFα was first identified as a 17 kD polypeptide in medium conditioned by activated monocyte-macrophages of both mouse and human origin (Aggarwal *et al.,* 1985). It is now known that TNFα can be produced by a variety of cell types including mononuclear phagocytes, T and B cells, mast cells and NK cells (Degliantoni *et al.,* 1985; Young *et al.,* 1987; Sung *et al.,* 1988; Steffen *et al.,* 1988; Liu *et al.,* 1989; Beutler and Cerami, 1989). The major biologically active form of TNFα has been shown to be a homotrimer of molecular mass 55 kD (Smith and Baglioni, 1987; Jones *et al.,* 1989).

Molecular forms

It was generally assumed that the secretion of TNFα was not unusual, as the intracellular precursor molecule contained a standard signal peptide, albeit one of unusually long amino acid sequence. The possibility that the long signal peptide might serve another function was suggested by Muller and coworkers (Muller *et al.,* 1986). It was subsequently shown (Muller *et al.,* 1986; Kriegler *et al.,* 1988) that TNFα protein can also exist as a 26 kD propeptide form (17 kD plus the signal peptide) which can be an integral membrane-bound protein which retains biologic activity (Decker *et al.,* 1986; Kriegler *et al.,* 1988; Luettig *et al.,* 1989; Perez *et al.,* 1990). Additional, novel, forms of cell-associated TNFα have been reported, produced in rodent cells (Young *et al.,* 1987; Bakouche *et al.,* 1988; Luettig *et al.,* 1989; Liu *et al.,* 1989; Kinkhabwala *et al.,* 1990; Keogh *et al.,* 1990).

Unlike human TNFα, the mouse protein can be glycosylated (Green *et al.,* 1976; Pennica *et al.,* 1984). Thus, reports of different molecular weight forms strongly suggested that, in mouse, TNFα can be produced in a variety of forms, which may reflect variable glycosylation patterns. Alternatively, these different isoforms may reflect different post-transcriptional or post-translational processing not related to glycosylation, such as differences in phosphorylation or sulfate patterns. Recent evidence using a macrophage cell line shows that mouse TNFα propeptide exists as multiple isoforms having molecular weights of 26 kD, 29 kD and 32 kD (Jue *et al.,* 1990; Sherry *et al.,* 1990). Furthermore, these isoforms result from differential glycoslyation (Jue *et al.,* 1990; Sherry *et al.,* 1990). The significance of these variable forms is not known.

It is now clear that TNFα is not actually secreted from cells but is "released" from the producing cell after proteolytic cleavage of the presequence peptide. Using a macrophage cell line, released TNFα has been shown to exist as multiple isoforms of 17 kD, 18.5 kD, and a "ladder" of approximately 6 glycosylated isoforms of molecular weight 21-25 kD (Beutler and Cerami, 1986; Cseh and Beutler, 1989; Jue *et al.*, 1990). The 18.5 kD isoform is not glycosylated and has a 10 amino acid N-terminal extension which exactly corresponds to the propeptide sequence (Cseh and Beutler, 1989). Apparently, this is the result of alternative propeptide processing. Furthermore, the 18.5 kD isoform is nonfunctional for inducing cytolysis of L929 TNF-sensitive fibroblast target cells (Cseh and Beutler, 1989). The function of this isoform is presently unknown.

Molecular biology

The mouse TNFα transcript of 1.6 kb contains an open reading frame of 235 (233 in human) amino acids of which 79 (76 in human) amino acids constitute a presequence, not present in the mature released TNFα (Pennica *et al.*, 1984; Pennica *et al.*, 1985). An additional transcript of 2.5 kb as been described (Michishita *et al.*, 1990; Descoteaux and Matlashewski, 1990), detectable after stimulation with LPS (Michishita *et al.*, 1990) or TNFα itself (Descoteaux and Matlashewski, 1990). Although TNFα protein has not yet been shown to be translated from the higher molecular weight transcript, these results suggest that different TNFα proteins may be generated, possibly by post-transcriptional modification of the mRNA by alternative splicing. However, it remains to be determined if multiple TNFα transcripts may exist under different stimuli and, if so, whether these transcripts play a role in the production of different forms of TNFα protein.

Human TNFα has 79% homology at the amino acid level with mouse TNFα, with an even higher homology (87%) in the prepeptide sequence (Pennica *et al.*, 1985). TNFα is also homologous (28% at the amino acid level, 46% at the nucleotide level) with lymphotoxin, also termed TNFβ, a cytokine produced by T helper cells with many biological activities similar to those of TNFα (Gray *et al.*, 1984). TNFβ binds to the same receptors as does TNFα. The TNFα and TNFβ genes are probably derived from duplication of an ancestral gene. Indeed, the

TNFβ locus is situated immediately 5' of the TNFα locus, within the major histocompatibility complex.

Regulation of synthesis

TNFα is highly regulated at the transcriptional, post-transcriptional, and post-translational level (Beutler *et al.*, 1986; Cseh and Beutler, 1989; Han *et al.*, 1990; Sherry *et al.*, 1990; Jue *et al.*, 1990; Trinchieri, 1991, Zuckerman *et al.*, 1991; Beutler, 1992; Kruys *et al.*, 1992). Transcriptional activation of TNFα may involve *k*B-type enhancers (Collart *et al.*, 1990) and factors interacting with an MHC class II-like 'Y box' (Shaklov *et al.*, 1990). Recent evidence supports a role for *k*B-type enhancers in the transcriptional regulation of TNFα, but fail to support a role for the Y box sequence (Drouet *et al.*, 1991). However, these investigators only studied LPS-stimulated transcriptional regulation. Transcriptional regulation of TNFα production by stimuli other than LPS has not been addressed. Post-transcriptional regulation is largely controlled by the 3'-untranslated UUAUUUAU octanucleotide repeating motifs which are capable of suppressing the translation of mRNA molecules in which they are expressed (Shaw and Kamen, 1986). More recently, Han and colleagues (Han *et al.*, 1990) using chloramphenicol acetyltransferase (CAT) coding sequences, demonstrated that downstream sequences present in TNFα mRNA are sufficient to mediate induction of CAT synthesis in response to activation by LPS. Thus, the 3'-untranslated region of TNFα mRNA can function to control both the half-life of the TNFα mRNA, thus preventing inappropriate persistent production of the protein, but also can function to markedly enhance the translational rate of TNFα protein production from mRNA. Although these studies are exciting, they have been done using only LPS activation of TNFα production. Whether these transcriptional controls for TNFα will extend to other inducers of this molecule remain to be determined.

TNF receptors

Specific cell-surface receptors for TNFα , both high- and low-affinity binding, are found on a variety of cells, including cells of the mononuclear phagocyte lineage (Imamura *et al.,* 1987). Early workers found that there are at least two forms of the TNF receptor, 80 kD and 60 kD (Smith and Baglioni, 1987; Thoma *et al.,* 1990; Hohmann *et al.,* 1990;) but the receptor can be variably glycosylated resulting in a variety of species from 46 kD to 85 kD It was suggested that the 60 kD species is sufficient for actively transducing the cytotoxic signal (Thoma *et al.,* 1990).

The TNFα receptors for both the human and mouse have now been cloned and expressed (Smith *et al.,* 1990a; Gray *et al.,* 1990; Kohno *et al.,* 1990; Loetscher *et al.,* 1990; Schall *et al.,* 1990; Lewis *et al.,* 1991; Goodwin *et al.,* 1991). The encoded receptors, as expected, are able to bind both TNFα and TNFβ. The mouse 55 kD receptor, named TNF-R1, is 425 amino acids in length, is 64% identical to its human counterpart and has two binding affinities for TNFα of 0.1 nM and 10 nM (Lewis *et al.,* 1991). Only one mRNA transcript (2.6 kb) has been found for this receptor. The mouse TNF-R1 has been mapped to chromosome 4 (Goodwin *et al.,* 1991). The mouse 75 kD receptor, named TNF-R2, has 452 amino acids, is 62% identical to the human TNF-R2 and has a binding affinity for TNFα of 0.1 nM. Two transcripts of 3.6 kb and 4.5 kb have been described for this receptor (Lewis *et al.,* 1991). The mouse TNF-R2 has been mapped to chromosome 6 (Goodwin *et al.,* 1991). Although the overall homologies are similar, the locations of strong homology are distributed differently. TNF-R1 is most conserved in the extracellular domain while TNF-R2 is most conserved in the intracellular domain. The weaker homology in the TNF-R2 extracellular region may help to explain the species specificity of TNF-R2.

TNF-R1 is 28% homologous with TNF-R2, approximately the same homology as each shares with certain other proteins (see below). However, there is a complete absence of homology between the intracellular domains of the two TNF receptors. This suggests that each receptor utilizes a distinct signaling pathway (Lewis *et al.,* 1991). Furthermore, the structural features important for signal transduction remain to be identified for each receptor, although receptor aggregation (Tartaglia and Goeddel, 1992a) and phosphorylation on serine residues, at least on TNF-R2 (Tartaglia and Goeddel, 1992b), may be important.

The cysteine-rich extracellular domain of the 75 kD receptor was shown to have extensive sequence homology with five proteins, including human nerve growth factor and CD40. Of great interest was the finding that the TNFα receptor extracellular domain had high homology to a transcriptionally active open reading frame from Shope fibroma virus (Smith *et al.*, 1990a). The transmembrane regions and intracellular domains were distinct for each protein. Nonetheless, these results indicate that the TNFα receptor is a member of a new and distinct family of cytokine receptors.

Most cell types and tissues appear to express both TNFα receptors (Lewis *et al.*, 1991). Although the function of each receptor is still being studied, it appears that TNF-R1 may send a cytotoxic signal while TNF-R2 may send a proliferative signal. This is an area of intense research with numerous hypotheses. One group (Tartaglia and Goeddel, 1992) have suggested that at high TNFα concentrations both receptors are occupied while at low TNFα concentrations, TNF-R2, the higher affinity-binding receptor, plays an indirect role in TNF-R1 responses by binding TNF and then delivering it to the lower affinity TNF-R1.

Soluble and intracellular TNFα receptors

In addition to cell-surface receptors, soluble forms of TNFα receptor also are found (Gatanaga *et al.*, 1990). These shed receptors correspond to the cell-surface receptors (Nophar *et al.*, 1990; Kohno *et al.*, 1990) and can antagonize TNFα mediated responses. The work of Kohno and colleagues (Kohno *et al.*, 1990) argues for post-translational modification, probably proteolysis, since transfection with full length cDNA results in soluble receptor.

Finally, it has been shown that intracellular TNFα receptors can be three times the number of cell-surface receptors (Watanabe *et al.*, 1988). Furthermore, it has been demonstrated that intracellular TNFα receptors can bind ^{125}I-TNFα with a Kd value similar to that found for surface TNFα receptors (Watanabe *et al.*, 1988).

Signal transduction

Although considerable effort has been put forth to understand the post-receptor binding, signaling pathway for TNFα, the available data are difficult to interpret. This is because these studies have been obtained using a variety of cells that respond differently to TNFα. Therefore, I will not discuss these various pathways here but refer to the review by Camussi and coworkers (Camussi *et al.*, 1991) which contains a good overview of these studies.

TNF effects on cell cycle

TNFα has been previously shown to affect cell cycling status. In transformed mouse L cell fibroblasts, TNFα has been shown to cause cytostasis, arresting cells transiently in the G_2 phase of the cell cycle (Darzynkiewicz *et al.*, 1984). Likewise, in the human leukemic cell line HL-60, TNFα has also been shown to temporarily arrest cells in G_2 while later (24-48 hours) it delayed progression through the G_1 phase (Darzynkiewicz *et al.*, 1987). More recently, TNFα has been shown to wholly or partially abolish cell cycling of the CFU-S hematopoietic compartment (See Figure I-1), implying that TNFα may inhibit cell cycle in normal hematopoietic progenitor cell populations (Warren *et al.*, 1990). It is likely that this effect of TNFα on cell cycle of early hematopoietic cells is the basis for its radioprotective effects on these cells (Slordal *et al.*, 1989).

TNFα has also been shown to keep Langerhans cells (an epidermal relative of the mononuclear phagocyte which has Fc receptors, can act as an antigen presenting cell, but is incapable of phagocytosis) alive but in a functionally immature state (Koch *et al.*, 1990). This effect of TNFα on the viability of these cells coupled with other data (Chen *et al.*, 1990) may suggest that TNFα serves some survival function for certain cell types.

TNFα effects on hematopoietic cells

In addition to its cytotoxic and cytostatic effects on transformed and leukemic cells, TNFα has been shown to have growth modulatory effects on normal hematopoietic cells and to have a protective effect on hematopoietic stem cells subjected to lethal doses of τ irradiation (Slordal *et al.*, 1989). In these mouse studies the number of colony forming units, day 12 spleen, (CFU-S_{12}) per animal increased with higher doses of TNFα administered. In contrast, a number of investigators have shown TNFα to have strong growth inhibitory effects *in vitro* on early committed hematopoietic progenitor cells, including colony forming unit, granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM), CFU-GM, BFU-E and CFU-E (Broxmeyer *et al.*, 1986; Peetre *et al.*, 1986; Eliason and Vassalli, 1988; Murphy *et al.*, 1988). However, it remains unclear whether the inhibitory effects of TNFα on early bone marrow-derived hematopoietic progenitor cells is a direct effect or is mediated by accessory cells contaminating the experimental system. Indeed, *in vivo* studies of the effects of TNFα on hematopoiesis do not support some of the in vitro work.

In vivo infusion of TNFα, at levels which are not directly toxic, results in a markedly decreased erythroid pool with significant suppression of late-stage erythropoiesis leading to anemia in chronically treated mice (Johnson *et al.*, 1988; Ulich *et al.*, 1990). In contrast to its *in vitro* inhibitor effects on early hematopoietic progenitor development, *in vivo* TNFα seems to specifically suppress later erythroid cells. CFU-E production or release of reticulocytes from the bone marrow is decreased, even though BFU-E, CFU-GM, CFU-m, and colony forming unit, megakaryocyte (CFU-Meg) progenitors are all significantly increased (Johnson *et al.*, 1988; Ulich *et al.*, 1990). This effect results in a peripheral anemia. It has been suggested that differences between *in vitro* versus *in vivo* effects reported for TNF are due to variation in actual experimental conditions such as dosage of TNFα used, or to variability in indirect effects such as production of G-CSF, GM-CSF, IL-1 or IL-6 (Ulich *et al.*, 1990). Nonetheless, it seems certain that TNFα has a dramatic inhibitory effect on erythropoiesis whether the experiments are performed *in vitro* or *in vivo*. TNFα effects on the rest of hematopoiesis await further clarification.

Relevance to thesis

Macrophages are a significant source of TNFα production and express both TNF-R1 and TNF-R2 on their surface. When my thesis research was started, there was no information regarding the effects of TNFα on the proliferation of growth factor-responsive, adherent macrophages, although inhibitory effects of TNFα on hematopoietic colony formation had been reported. Thus, an examination of the response of mouse BMM to TNFα alone, and in combination with the three macrophage growth factors CSF-1, GM-CSF and IL-3 was undertaken to establish a possible role for TNFα in the regulation of the proliferation of relatively mature growth-competent macrophages. If exogenous TNFα can modulate the proliferation of mouse BMM, then it would be valuable to examine further the nature of the response, to better define the role of TNFα on cells of myeloid origin. An investigation as to the potential paracrine/juxtacrine/autocrine, positive or negative nature of the response may reveal important aspects of the regulation of macrophage proliferation. Since TNFα can be produced by macrophages and is highly regulated post-translationally, an examination of the molecular nature of the TNFα produced in BMM may allow an assessment of the potential for an autocrine role for TNFα in the proliferation of mouse macrophages. The earlier observations of intracellular TNF receptors capable of binding TNFα with similar Kd as surface receptors further suggests that TNFα may serve an intracrine function.

MULTI-FACTOR REQUIREMENTS FOR PROLIFERATION

The hypothesis of a two-factor requirement for optimal proliferation of mature, growth-competent mouse macrophages is not novel as other cell proliferation systems have been shown to have multifactor requirements for growth. Until fairly recently, it was generally thought that the control of myeloid hematopoiesis involved single factors acting on cells at specific stages of hematopoietic cell differentiation. For example, it has been suggested that IL-3 acts on BFU-E, the early erythroid progenitor cell, until a critical point is reached where the differentiating cell would now respond to the single cytokine Epo. However, early studies on other cellular systems (Temin, 1971; Pledger *et al.*, 1977; Scher *et al.*, 1979; Meyer *et al.*, 1979; Stiles *et al.*, 1979), concluded that a succession of cellular processes (or factors) is required to lead to DNA synthesis and full mitogenic responsiveness. It, therefore, seemed likely that, with further study, myeloid cells may also be shown to require the simultaneous actions of multiple factors to realize their full potential for proliferation and differentiation. Indeed, it is now clear that a much more complex situation exists for hematopoietic cell survival, growth and differentiation. Recently, many reports have emerged documenting the effects of combinations of growth factor cytokines, which act in an additive or synergistic fashion, on proliferation and development of early and late progenitor cells, as well as mature hematopoietic cells. The following are synopses of the literature on the simultaneous actions of growth factors on different cell types.

Fibroblasts

Pledger *et al.*, 1977 and Scher *et al.*, 1979, showed that human serum contains two sets of factors that function synergistically to promote growth of mouse Balb/c 3T3 fibroblasts. The first factor has been termed a "competence" factor and regulates the emergence of the fibroblast from a quiescence state (G_0). Platelet derived growth factor (PDGF) is the prototype cytokine but other cytokines can provide this competence step including fibroblast growth factor (FGF). Once fibroblast cells become competent, they may no longer require the competence factor and progression through later G1 and on to DNA synthesis requires another cytokine or "progression" factor (Scher *et al.*, 1979; Stiles *et al.*, 1979; Campisi and Pardee, 1984). Epidermal growth factor (EGF) is the prototype progression factor, but other cytokines can provide this progression step including insulin-like growth factors (IGFs) such as somatomedin C (Stiles *et al.*, 1979; Campisi and Pardee, 1984) or transforming growth factor-α, which can bind to the EGF receptor and transduce a signal. Growth factors are much less involved in passage through the remainder of the cell cycle (Pardee, 1992).

Lymphocytes

One of the T cell proliferative effects which requires two factors was so dramatic that it formed the basis for the discovery of IL-1 (Gery *et al.*, 1972).

This, of course, is the mouse thymocyte assay which requires costimulation using both a lectin and IL-1 for optimal proliferation.

It is now well established that T lymphocytes require at least two signaling events in order for these cells to initiate proliferation (Meyer *et al.*, 1984). One event involves the stimulation of the T cells with lectins, anti-CD3 antibodies or by cognate recognition by the T cell receptor complex of processed antigen in the context of MHC class II molecules on antigen presenting cells. This event serves to prime or activate the T cell, signaling transition from the resting G_0 state to the G_1 phase of the cell cycle, and can be compared to the competence stage in fibroblasts (Golub and Green, 1991). The second event involves the autocrine production of interleukin-2 (IL-2), in conjunction with the IL-2 receptor, which stimulates the cells to enter the S phase of the cell cycle and replicate. Thus, IL-2 serves as a progression factor for DNA synthesis to occur. These two events can be substituted with pharmacological agents such as calcium ionophores (competence) and phorbol esters (progression); neither of these agents can initiate DNA synthesis alone.

B lymphocytes, like T lymphocytes, also require at least two signaling events. One event involves antigen recognition by the immunoglobulin receptor of the B cell which apparently serves some competence function. The second event requires IL-2 and/or IL-4, produced by activated T cells, to act on the B cells and induce proliferation and differentiation (progression step).

Macrophages and macrophage progenitors

Successful stimulation of proliferation of macrophage progenitors in serum-free medium has been shown to require at least two cytokines (Guilbert, 1985): CSF-1 and GM-CSF or IL-3; serum can substitute for the costimulatory factor in this system. In contrast, proliferation of more mature macrophages, such as bone marrow-derived macrophages (BMM), requires only a single cytokine; either CSF-1, GM-CSF or IL-3 (Guilbert, 1985; Branch and Guilbert, 1986; Chen *et al.*, 1988), even in serum-free systems (Guilbert, 1985). However, the maximal proliferative response of macrophages to these individual cytokines can often be enhanced when these factors are mixed together or by mixing experiments with additional factors (Hamilton *et al.*, 1988). These positive cooperative effects can be additive or more than additive, termed synergistic (see Cooperative interactions, below).

Cooperative interactions

Bradley and Hodgson (Bradley and Hodgson, 1979) were the first to report on a cooperative synergy factor which, when combined with CSF-1, resulted in the formation of giant macrophage colonies from very immature bone marrow progenitor cells. Subsequently, other investigators reported on the cooperation of pure or recombinant factors to increase the growth of purified mouse granulocyte-macrophage progenitor cells (Williams *et al.*, 1987a), as well as increased production of HPP-CFC after *in vivo* administration of CSF-1 plus IL-3 (Williams *et al.*, 1987b). Since these earlier publications, numerous reports of cooperative effects of hematopoietic growth factors on the stimulation, both *in vitro* and *in vivo*, of early primitive stem cells and early progenitors have emerged. Indeed, these combinations seem endless: GM-CSF synergizes with G-CSF, IL-3 and CSF-1; G-CSF synergizes with IL-3 and CSF-1; IL-6 synergizes with IL-4, G-CSF, GM-CSF, CSF-1, and IL-3; IL-3 synergizes with CSF-1; IL-1 synergizes with CSF-1; (McNiece *et al.*, 1982; Bartelmez and Stanley, 1985; Williams *et al.*, 1987a; Quesenberry *et al.*, 1987; Bartelmez *et al.*, 1989; McNiece *et al.*, 1988; McNiece, 1990; Iscove and Yan, 1990). In addition, Chen *et al.*, 1988, has reported on the synergistic effects of GM-CSF with CSF-1 on more mature bone marrow-derived macrophages, monocytes and peritoneal macrophages. However, with BMM, the system used by Chen *et al.*, probably was measuring the growth and proliferation of macrophage progenitors rather than more differentiated macrophage populations. Nonetheless, it appears that optimal conditions for the *in vitro* proliferation of hematopoietic cells may require more than a single cytokine. Indeed, in some experimental systems a trimolecular synergy has been noted for optimal growth of cells (Bartelmez *et al.*, 1989).

Relevance to thesis

Based upon the requirements for both competence and progression signals for entry into S phase and replication for other cell types, it seems reasonable and likely that macrophages would have a similar requirement. The possibility of multiple factor requirements for mouse macrophage proliferation is examined in this thesis. Indeed, the data thus far on the mitogenic requirements for a number of cell systems, both hematopoietic and nonhematopoietic, indicate that, in nature, cells require multiple signals for optimal growth. Multiple signaling requirements for normal growth makes sense since this implies that a single mutational event would not, necessarily, result in abnormal growth behavior. It assumes that multiple "hits" would be required, thus, lessening the chances of malignant transformation. However, the current dogma for macrophage proliferation is that these cells may require only CSF-1 for optimal proliferation. This thesis examines whether this dogma is correct. Thus, in part, this thesis identifies which, if any, cytokines are capable of cooperating with CSF-1, to increase the proliferative potential of normal mouse bone marrow-derived macrophages and macrophage cell lines. Multiple cytokine signaling may be either simultaneous, i.e., acting together most probably within the cell cycle, or in series, i.e., acting at different stages of the cell cycle such as competence and progression factors. Thus, identification of multifactor combinations for optimal growth may indicate dual or complementary signaling pathways which are essential or important for optimal macrophage proliferation.

AUTOCRINE GROWTH REGULATION

Stimulation of cells by autocrine mechanisms is an emerging concept in the search for the cellular and molecular basis of malignant transformation. It has been known for many years that, *in vitro*, malignant cells require less exogenous growth stimulatory agents than do normal, nontransformed cells (Holley, 1975). These observations led to the hypothesis that malignant cells produce growth factors endogenously and this autocrine production contributes to, or is responsible for, the malignant transformation. Sporn and Todaro, 1980, have termed this mechanism "autocrine secretion." Support for this hypothesis has increased over the years, particularly with the emergence of the relationship of many oncogenes and proto-oncogenes with growth factors or growth factor receptors (Roberts *et al.*, 1985; Pierce, 1989).

Although much effort has been directed at showing the relationship of autocrine secretion with growth of malignant cell phenotypes, transformed cell lines, and transfected cell lines, very little data, with the notable exception of the lymphoid system (Golub and Green, 1991; see above Multi-Factor Requirements for Proliferation), has been presented indicating any role for autocrine stimulatory mechanisms in normal cellular development.

Transformed or malignant cells

The production of growth factors by transformed cells has led to the suggestions that autocrine growth stimulation may be of importance for the maintenance of the transformed phenotype (Sporn and Toduro, 1980; Sporn and Roberts, 1985). Experimental proof for autocrine growth stimulation is mostly circumstantial or correlative. Such evidence has been difficult to obtain, in part because many cells which synthesize and secrete growth factors generally appear to lack the corresponding receptors at the cell surface. This may, however, represent down-regulation of the receptors by endogenously produced ligand which binds to the receptor intracellularly or immediately upon release, effectively blocking available sites for exogenous ligand and/or resulting in the rapid internalization of the ligand-receptor complexes. However, there are examples of transformed cells where autocrine growth mechanisms have been linked to the abnormal growth of these cells. One of the best examples studied are mechanisms involving PDGF (see Ross *et al.*, 1986 for a review). PDGF is a dimer comprised of A and B chains, the B chain gene is the normal cellular homologue to the oncogene v-*sis* of simian sarcoma virus (Ross *et al.*, 1986). The genes for PDGF and the PDGF receptor have been shown to be present and expressed in human malignant glioma cell lines (Hermansson *et al.*, 1988) and human osteosarcoma cell lines (Betsholtz *et al.*, 1986).

The major body of work that supports the concept of autocrine growth mechanisms has been done *in vitro* by insertion of growth factors or growth factor receptors into cells that express the complementary component. Of interest is that tumorigenicity can be demonstrated in cell lines that are already immortalized. In contrast, where normal primary cells were manipulated to express autocrine growth, conversion of these autonomously growing cells to the transformed phenotype could not be demonstrated (Lang and Burgess, 1990). However, cells from patients with malignant diseases have been shown to be, at least in part, autocrine. Thus, studies using neutralizing antibodies to growth factors have indicated a potential autocrine role for IL-6 (Yee *et al.,* 1989; Kawano *et al.,* 1989) and IL-1 (Sakai *et al.,* 1987) in neoplastic growth.

Virus-infected or transfected cells

Autocrine growth has been shown to occur in growth factor-dependent cells which have either the receptor but lack the ability to produce the growth factor or produce the growth factor but lack the receptor. After infection or transfection with the component which is lacking, these cells proliferate in the absence of exogenously added growth factor. It has been shown for a number of cytokines (GM-CSF, IL-2, IL-3, IL-5, IL-6, IL-9) that the autocrine stimulation of these transfected cells correlates with malignant transformation (Lang *et al.*, 1985; Yamada *et al.*, 1987; Wong *et al.*, 1987; Blankenstein *et al.*, 1990; Vink *et al.*, 1990; Uttenhove *et al.*, 1991) Fibroblasts after transformation with simian sarcoma virus containing the oncogene v-*sis* (the expressed protein is the homologue to the PDGF B chain) become transformed and grow autonomously. The growth of these cells can be arrested using antibodies to PDGF (Huang *et al.,* 1984).

Normal cells

There are few reports documenting autocrine growth mechanisms in normal cells although autocrine mechanisms affecting cell differentiation have been described (Golub and Green, 1991; Rieckmann *et al.*, 1991). Endogenous production of CSF-1, GM-CSF, G-CSF, TNFα, IL-1 and IL-6 has been shown to occur in normal macrophages (Sullivan *et al.,* 1983; Aggarwal *et al.,* 1985; Metcalf and Nicola, 1985; Fibbe *et al.,* 1986; Horiguchi *et al.,* 1986; Horiguchi *et al.,* 1987; Oster *et al.,* 1987; Rambaldi *et al.,* 1987; Cannistra *et al.,* 1987; Durum and Oppenheim, 1989;). However no autostimulatory role for any of these cytokines has been documented despite the presence of the corresponding receptor on these cells and the fact that macrophages proliferate to exogenous CSF-1 and GM-CSF and these factors can induce each other (Horiguchi *et al.,* 1987). Thus, there are no reports which document an autocrine growth mechanism operative in normal hematopoietic cells of myeloid lineage, although autocrine mechanisms regulating function have been reported (Nacy *et al.,* 1990). However, it is significant that of the reports documenting the cooperative effects of mixing exogenous cytokines with CSF-1, all have shown that these factors are cytokines which can be produced by macrophages. Chen *et al.,* 1988, have shown that suboptimal levels of GM-CSF and optimal levels of CSF-1 cooperate to increase the growth of peritoneal exudate macrophages, blood monocytes and bone marrow-derived macrophages (BMM) above that produced by either factor alone. However, the system used in these studies (whole bone marrow grown in culture without selection for mature macrophage populations) to examine BMM probably was evaluating the proliferation of macrophage progenitor cells rather than more mature macrophages. Thus, the growth factor requirements for mature, adherent BMM remain uncertain.

Autocrine stimulatory mechanisms potentially operative in normal cells

One can postulate at least three mechanisms (Browder *et al.,* 1989; Logan 1990) by which normal cells may regulate their own growth or function. One mechanism would be an external, or "public", loop involving endogenous production and secretion of a mitogenic cytokine for which the producing cell has the corresponding receptor expressed on its cell surface. This would involve normal ligand-receptor interaction with internalization through normal endocytic pathways, and transduction of a mitogenic signal.

Another mechanism would be internal, or "private", occurring within the cytosol of the cell, involving endogenous production of a mitogenic cytokine which binds to its receptor within the cytosol, most probably within the endoplasmic reticulum or Golgi apparatus (Dunbar *et al.*, 1989), and can transduce a mitogenic signal.

A third possible mechanism would involve an endogenously produced cytokine which would be capable of sending a mitogenic signal within the producing cell, without first binding to its receptor. Thus, the cytokine remains within the cell of origin and acts directly as an intracellular messenger. This mechanism has been referred to as "intracrine" (Logan, 1990). This mechanism may involve cytokines which do not have classical signal peptide sequences to direct their secretion. These cytokines would include IL-1α, IL-1β, some of the fibroblast growth factors, and others (March *et al.*, 1985; Oppenheim *et al.*, 1986; Jaye *et al.*, 1986; Abraham *et al.*, 1986). TNFα may also qualify as a cytokine potentially capable of intracrine interactions. Its signal peptide is unusually long, is extremely conserved between animal species, and, an important, but as yet undefined, biological role for the signal sequence has been postulated (Pennica, 1985; Kriegler *et al.*, 1988; Perez *et al.*, 1990).

For simplicity, I will only use the terms autocrine and intracrine, meaning, respectively, an external or "public autocrine" loop and an internal "private autocrine" loop or "intracrine" mechanism. These autocrine or intracrine mechanisms can be of a constitutive nature, or secondary, mediated by an external signal, such as occurs for T lymphocytes (Golub and Green, 1991). Validation of a particular mechanism can be obtained by using neutralizing antibody to the cytokine (or antibody to the receptor) to validate autocrine mechanisms. Microinjection of neutralizing antibody may help to validate either autocrine or intracrine mechanisms. Blocking of protein production, either at the transcriptional or translational level, using antisense or gene "knock-out" approaches, could be used regardless of the mechanism of autostimulation. However, microinjection of neutralizing antibody or blocking of the ligand mRNA translation product, although helpful to confirm either autocrine or intracrine mechanisms, would not differentiate between these.

Relevance to thesis

In various qualitative and quantitative degrees, any of the three cytokines which stimulate macrophage proliferation on their own (CSF-1, GM-CSF or IL-3), are reported able to induce cytokine production from mononuclear phagocytes and are, themselves produced by macrophages (see Myeloid growth factors, above). In addition, other cytokines which can be produced by macrophages such as IL-1 and TNFα have been shown to have hematopoietic regulatory activities (Oppenheim *et al.,* 1986; Broxmeyer *et al.,* 1986). Thus, the difference in the number of cytokines required for proliferation of macrophage progenitors (two or more; see Multi-Factor Requirements for Proliferation above), or for mature macrophages (only one) may lie in the ability of the more mature cell to produce its own accessory growth factor in response to a primary mitogenic stimulus. This autocrine or intracrine "second signal" could be any of the myeloid growth factors or other cytokines produced by macrophages such as IL-1 or TNFα.

It makes sense that individual cells would possess some mechanism(s) for regulating their own growth, since dependence solely on exogenous regulatory mechanisms could result in the loss of homeostasis. Autocrine control could be accomplished by negative feedback mechanisms, i.e., secondary autocrine production of growth-inhibitory factors. However, another redundancy for control of cellular growth would be a requirement for autocrine production of a growth-stimulatory factor that, ideally, would be essential for the complete growth response signal.

Thus, for example, in an analogous situation as for lymphocytes, where TcR-complex recognition of MHC/antigen acts to make the cells competent for the progression signal supplied by endogenously produced IL-2 (resulting in a proliferative response), in macrophages, CSF-1 may serve a similar competence function and a secondarily generated endogenous factor may serve as the signal for progression through the cell cycle. Conversely, an endogenously produced cytokine may serve a competence function while an exogenous stimulus supplies a progression signal.

RATIONALE FOR THESIS

This thesis, in general, examines regulation of normal macrophage proliferation. It is likely that the proliferation of mononuclear phagocytes is regulated by a complex interaction of both positive and negative signals sent by soluble (humoral) molecules termed cytokines. Based on the results of previous workers using *in vitro* colony formation in semi-solid agar cultures and proliferation of more differentiated, adherent cells in tissue culture assays, a dogma has arisen that the cytokine CSF-1, the macrophage-specific colony-stimulating factor (M-CSF), is the major mitogenic agent for the survival, growth, function, and differentiation of mouse and human mononuclear phagocytes, and that it acts alone. However, the development of mouse and human macrophage progenitors requires at least two stimuli, CSF-1 plus either IL-3 or GM-CSF suggesting that CSF-1 alone does not provide a complete mitogenic signal to these cells.

Proliferation of more differentiated, adherent mouse monocyte-macrophages appears to require only a single mitogenic agent (either CSF-1, GM-CSF or IL-3), even when using serum-free medium. The difference between the growth factor requirements of macrophage progenitor cells (at least two factors) and more differentiated macrophages (one factor) may simply reflect differences in the levels of growth factor receptors expressed on these cells. CSF-1 receptor levels are 10-fold higher on mature growth-competent macrophages as compared to early committed progenitor cells. Alternatively, it is possible that CSF-1 indirectly stimulates more differentiated macrophages to proliferate by inducing the internal production of an endogenous growth-relevant cytokine(s). The ability of mononuclear phagocytes to produce cytokines may increase as these cells become more differentiated. Indirect support for this comes from the high levels of cytokines such as TNFα, IL-1, and IL-6 which may be produced by mature effector macrophages during an inflammatory response. Endogenously-derived cytokines may act by autocrine or intracrine mechanisms to also affect proliferation. Thus, the ability of CSF-1 alone to stimulate proliferation of mature cells could be due to either an enhanced number of CSF-1 receptors, leading to greater overall mitogenic stimulation, or to the ability of mature cells to provide accessory cytokines endogenously. In the latter case, CSF-1 may not provide a complete mitogenic signal. Thus, endogenously produced cytokines may be obligatory for the observed proliferative response to exogenously added mitogenic agents or they may be complementary, serving to augment the proliferation of the cells.

Good model systems for examining the growth factor requirements of mouse macrophages *in vitro* are not readily available. Thus, an added problem to the study of the regulation of macrophage survival, growth, proliferation and differentiation has been that many of the CSF-1 studies, prior to my thesis work, have examined progenitor cells from whole bone marrow cells, or studied BMM without providing adequate evidence of the homogeneity of the macrophage population, i.e., morphology, cell surface markers, phagocytosis, nonspecific esterase, etc. Certain studies (Tushinski *et al.,* 1982; Tushinski and Stanley, 1985) showed >95% CSF-1R bearing cells as evidence for homogeneity. However, CSF-1R is found on a number of cell types, both hematopoietic and nonhematopoietic (see CSF-1 above) and thus, used alone this criterion may be misleading. Therefore, because of the potential heterogeneous nature of these experimental systems, previous studies have the caveat that the cytokines being used may not be acting directly on the cells under study. Thus, it remains uncertain as to which cytokine(s) act alone, or in cooperation, to induce macrophage proliferation. Although cell lines have been useful for examining certain of these aspects, cell lines are not normal cells, and data collected using cell lines must be tempered with questions regarding the normal physiologic relevance of the results. Thus, a degree of uncertainty exists regarding the extent to which CSF-1 (and other mediators) acts as a mitogenic agent for normal macrophages.

An understanding of the normal proliferative responses of macrophages may lead to a better understanding of neoplastic growth. In this regard, better definition of the relative roles of cytokine effects on macrophage proliferation may allow a clearer understanding of the complex network of interdependent interactions involved in the regulation of normal macrophage growth. This is of importance in order to understand possible disregulations in transformed counterparts. Furthermore, before examining mechanisms of action for CSF-1, or other stimulators of macrophage survival, growth, proliferation or differentiation, it would be of importance to understand whether the function attributed to the cytokine in question, i.e., proliferation, enhanced cytotoxicity against tumors, etc., is a direct effect or whether the effect is mediated fully or partially by the secondary induction of an endogenous mediator.

MAJOR THESIS OBJECTIVE & WORKING HYPOTHESIS

The cytokine CSF-1 appears to be the major mitogenic agent stimulating the *in vitro* proliferation of mouse macrophages. However, it is presently unknown whether CSF-1 acts alone or requires obligatory co-stimulatory factors for the stimulation of proliferation of normal, growth-responsive mouse macrophages. The major objective of my thesis is to determine the growth-stimulatory factor requirements for the proliferation of mature, growth-responsive, adherent mouse monocyte-macrophages, whether these cells require multiple mitogenic signals for optimal proliferation, and whether endogenous cytokine production contributes to the observed proliferative response to external stimuli. A working hypothesis is that macrophages require at least two mitogenic signals, one of these signals is provided by an external, paracrine (or endocrine), stimulus while the second signal is internally generated and autocrine, being induced by the first stimulus.

HYPOTHESIS I: *OPTIMAL PROLIFERATION OF NORMAL MOUSE MACROPHAGES REQUIRES STIMULATION BY AT LEAST TWO FACTORS, ONE OF WHICH IS A MACROPHAGE GROWTH FACTOR (CSF-1, GM-CSF, IL-3).*

SPECIFIC OBJECTIVES

1. **To isolate and characterize a growth factor-dependent mouse macrophage cell line.**

2. **To isolate and characterize homogeneous populations of primary, growth factor-dependent mouse macrophages.**

3. **To determine which lympho-hematopoietic cytokines stimulate macrophage proliferation.**

4. **To determine which cytokines, if any, modulate cytokine-stimulated macrophage proliferation.**

APPROACH TO SPECIFIC OBJECTIVES

Objective 1: To isolate and characterize a growth factor-dependent mouse macrophage cell line.

A growth factor-dependent macrophage cell line (S1) was subcloned from a v-*myc*-transformed growth factor-responsive C3H/HeJ-derived line (5/10.14). This cell line after additional cloning was compared to normal, primary BMM for similarity in regard to morphology, cell surface antigen expression and response to mitogenic agents (see Chapters II and III).

Objective 2: To isolate and characterize homogeneous populations of normal, primary growth factor-dependent mouse macrophages.

Proliferation of mature 'young' mouse macrophages (just beyond the monocyte stage) can be studied *in vitro*. These cells can be obtained in high yield as relatively homogeneous populations. Thus, methods were developed for the preparation of pure populations of primary bone marrow-derived macrophages (BMM), from progenitors collected from the femurs of C3H/HeJ and C3H/HeN mice. The method maximized macrophage homogeneity and minimized contamination by non-macrophage cells, particularly fibroblasts (see Chapter III).

Objective 3: To determine which lympho-hematopoietic cytokines stimulate macrophage proliferation.

More than 30 factors capable of affecting hematopoietic cell growth have been cloned and expressed. Not all of these molecules were examined for their effects on macrophage proliferation. Initially, it seemed reasonable to examine the five myeloid growth factors (CSF-1, GM-CSF, G-CSF, IL-3 and Epo) as these factors control the production of myeloid lineage cells. In addition, since autocrine regulation was being considered, macrophage products were examined for direct and modulatory mitogenic actions. In particular, I chose to focus on those factors that had previously been shown to have effects on hematopoietic cells. These included, the myeloid growth factors, CSF-1, GM-CSF, G-CSF, Epo, as well as TNFα, TNFβ, IL-1α, IL-6, TGFβ and PGE_2. Whether any of these molecules stimulated proliferation of mouse macrophages was examined (see Chapters II, III and Appendix 1a-1j).

Objective 4: To determine which cytokines, if any, modulate cytokine-stimulated macrophage proliferation.

Using the same agents described above, under objective 3, we examined whether any of these molecules had positive or negative modulatory effects on mouse macrophage proliferation stimulated by the major macrophage growth factors, particularly CSF-1 (see Chapters II, III and Appendix 1a-1j).

The studies under objectives 3 and 4 were carried out with both BMM and the S1 macrophage cell line. The rationale for using both primary cells and a cell line was that, if an activity is observed with both the cell line and the primary cell populations, it is unlikely to be due to a secondary effect mediated by non-macrophage accessory cell contamination. Also, it was reasonable that the use of C3H/HeJ animals would increase the chances of observing cooperative responses because these animals have a genetic defect which renders these animals unresponsive to the potentially growth modulating effects of lipopolysaccharides (LPS), a commonly found contaminating molecule in culture systems. Furthermore, this defect in LPS response also appears to control production of a number of endogenously produced cytokines, rendering these animals cytokine hypoproductive to LPS stimulation. Whether this defect applies to other stimuli will also be examined in this thesis.

Experimental Questions Considered under Hypothesis I

Question 1: Is CSF-1 the major mitogenic agent for macrophages?
See Chapter II, III, and Appendix 1a-1j.

Question 2: Are there cytokines that cooperate with macrophage growth factors to increase the maximal mitogenic signal?
See Chapters II, III, and Appendix 1a-1j.

Question 3: Are there cytokines that have negative effects on the proliferation of mouse macrophages?
See Appendix 1a-1e, 1i-1j.

Question 4: Do exogenous cytokines modulate the binding of CSF-1 to its receptor?
See Chapter III and Appendix 1k.

Question 5: Do exogenous cytokines stimulate the endogenous production of growth-relevant cytokines from macrophages?
See Appendix 1l-1s.

Of the cytokines examined, CSF-1 appeared to be the major mitogenic agent for the proliferation of mouse macrophages. Furthermore, only TNFα (also TNFβ, which binds the same receptor) showed a cooperative growth-stimulatory effect with CSF-1, but not GM-CSF or IL-3, on both BMM and the S1 macrophage cell line. Binding of CSF-1 to its receptor was found to be down-regulated by many different agents, including CSF-1, GM-CSF, TNFα, IL-3, PGE$_2$, and albumin. Most agents induced the secretion of low levels of CSF-1 from BMM and the S1 cell line. However, neutralizing anti-CSF-1 indicated that this level of exogenously released CSF-1 was unimportant for GM-CSF-stimulated growth of normal cells. CSF-1 was shown to induce TNFα but not IL-1 or IL-6, while the combination of GM-CSF and TNFα was the best at inducing IL-1 production. Endogenous production of GM-CSF was only found after CSF-

1 stimulation. However, experiments using neutralizing anti-GM-CSF indicated that the production of GM-CSF by BMM was not sufficient to affect the CSF-1-mediated proliferation of these cells. TNFα was, thus, the only agent identified which showed cooperativity with CSF-1 for the proliferation of mouse macrophages, decreased CSF-1 receptor levels and is produced by macrophages. Therefore, it is a potential autocrine factor for modulating the proliferation of mouse macrophages. These observations led to the formulation of a second hypothesis.

HYPOTHESIS II: *ENDOGENOUS PRODUCTION OF TNFα IS ESSENTIAL (OR IMPORTANT) FOR MITOGENIC STIMULATION OF MOUSE MACROPHAGES.*

Specific Objective under Hypothesis II: To determine whether TNFα acts as an endogenous autocrine stimulatory molecule.

To ascertain whether the macrophage populations that cooperatively respond to TNFα can produce TNFα, two approaches were tried: (1) TNFα messenger RNA levels were determined before and after stimulation by CSF-1 (see Chapter V); and (2) protein levels of TNFα, both cell-associated and released forms, were determined by immunoprecipitation of biosynthetically labeled TNFα translation product and by specific bioassay (see Chapter V). In order to quantitate levels of TNFα protein produced by the macrophage populations, it was necessary to develop a more sensitive TNFα bioassay than currently existed (see Chapter IV). Also, methods were developed to allow for microimmunoprecipitation of TNFα proteins from only 10^6 starting cells (see Chapter V).

In order to determine whether endogenously produced TNFα functions as an autocrine and/or intracrine agent, two approaches were tried: (1) Neutralizing anti-mouse TNFα antibody to block a possible autocrine loop (see Chapter V); and (2) Synthetic oligodeoxynucleotides made antisense to TNFα to specifically inhibit total cellular production of TNFα to block either an autocrine or intracrine loop (see Appendices 2 and 3). Since TNFα synthesis has to be inhibited for >24 hours to assess the effects on cellular proliferation, phosphorothioate oligodeoxynucleotides (S-oligos) were chosen over unmodified or methlyphosphonate reagents because of their relative ease of entry into cells coupled with their high resistance to degradative enzymes.

Experimental Questions Considered Under Hypothesis II

Question 1: **Is TNFα mRNA constitutively present, or inducible by CSF-1, in primary growth-competent mouse macrophages or macrophage cell lines?** See Chapter V

Question 2: **Is TNFα protein detectable prior to, or after, mitogenic stimulation?** See Chapter V.

Question 3: **What effect does neutralizing anti-mouse TNF have on CSF-1/GM-CSF-mediated macrophage proliferation?** See Chapter V.

Question 4: **Can translation of TNFα protein be inhibited by S-oligos made antisense to TNFα mRNA?** See Chapter VI and Appendix 2.

Question 5: **What effect does S-oligo antisense inhibition of intracellular TNFα protein have on the proliferation of mouse macrophages to macrophage growth factors, particularly CSF-1?** See Chapter VI and Appendix 3.

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CHAPTER II

REGULATION OF HEMATOPOIESIS BY GROWTH FACTORS:

Proliferation of the mouse macrophage as a model for stimulatory and inhibitory effects[1]

OVERVIEW

This chapter contains a general overview of the control of hematopoietic cell development by soluble growth stimulatory and inhibitory factors, demonstrates multiple factor requirements for optimal stimulation of proliferation of a mouse macrophage cell line, and provides justification for using BMM as a model system for the study of the relative roles of hematopoietic growth factors on the proliferation of these cells. The S1 macrophage cell line, isolated from a v-*myc*-transformed macrophage cell line of C3H/HeJ bone marrow origin, is shown to be growth factor-dependent (Table II-3). These cells proliferate to either CSF-1, GM-CSF or IL-3 alone in serum-containing medium. However, these cells show cooperative proliferative responses when CSF-1 is combined with GM-CSF but not with G-CSF, and not when GM-CSF is combined with IL-3 (Figures II-2 and II-3). Moreover, when CSF-1 is combined with the cytokine IL-1α, a cooperative synergism is observed (Figure II-4). Thus, these results address specific objective 1 of the thesis, the isolation of a growth factor-dependent macrophage cell line. Moreover, the data provide justification for using mouse macrophages derived from bone marrow as a model system for the examination of mitogenic effects.

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[1] A version of this chapter has been published. Guilbert, L.J. and Branch, D.R. (1989) Regulation of Hematopoiesis by Growth Factors: Proliferation of the murine macrophage as a model for stimulatory and inhibitory effects In: Immune Consequences of Trauma, Shock and Sepsis (Eds. Faist, Ninnemann & Green), Berlin Heidelberg: Springer Verlag.

INTRODUCTION

Sepsis is the major cause of late deaths after thermal or traumatic injury (Miller *et al.*, 1982). Central to host resistance to sepsis is maintenance of the levels and functions of immune cells: granulocytes, monocytes, and lymphocytes, all of which are of hematopoietic origin. Among the many documented alterations in the function and levels of blood cells following thermal injury are anemia (Wallner and Vantrin, 1986), loss of neutrophil chemotaxis (Nelson *et al.*, 1986), immediate neutrophil leukocytosis (McIver, 1933) and delayed granulocytopenia (Asko-Seljavaara, 1986). Many of the elements that regulate function of blood cells also regulate production (Warren and Ralph, 1986). Thus, sepsis following trauma can be viewed, in part, as a consequence of dysfunction in the regulation of hematopoiesis.

It is the purpose of this article to introduce and review hematopoiesis from the point of view of control by soluble (humoral) factors and then to illustrate the complex and interacting nature of humoral control with results from our laboratory that examine a well-defined experimental model. The review is focused on the myeloid arm of blood-cell formation which includes production of erythrocytes, the granulocytes (neutrophilic, eosinophilic, basophilic), monocytes, and platelets. The experimental data presented is focused even further in examining the regulation of the growth of macrophages in culture.

Hematopoiesis

Blood cells, both myeloid and lymphoid, all derive from a common class of self renewing, pluripotent stem cells (reviewed in Metcalf, 1984, see Figure II-1). These cells give rise to myeloid hematopoietic stem cells which also have large replicative ability but which have lost the potential to produce lymphocytes. Maturation at this very immature cell stage proceeds with continued loss of potential, both in terms of the numbers and types of cells that individual progenitor cells can produce. Eventually, progenitor cells restricted to producing a single blood-cell type arise, marking the origin of specific differentiation lineages. These unipotent progenitor cells continue to mature giving rise to the morphologically identifiable hematopoietic cells and finally to nongrowing, functional blood cells. Regulated proliferation accompanies differentiation at all stages.

Regulation of hematopoiesis

Proliferation is regulated at multiple levels. There is both positive and negative control, the control elements can operate over long range (greater than 10 cell diameters, endocrine) or short range (cell-cell contact, paracrine or autocrine), and the elements can target cells of widely differing maturities (Tables II-1 and II-2).

Inhibitors of hematopoiesis

Although maintenance of stem cells in a quiescent state by inhibitors or cell-cell contact (reviewed in Toksoez *et al.,* 1980) has long been an accepted dogma, little is known of the mechanism of inhibition of stem cell proliferation. Recently, however, transforming growth factor beta (TGFβ) has been shown to be a selective and potent inhibitor of the development of only the very immature progenitor cells such as the colony-forming unit - granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM; Keller *et al.,* 1988). Other well defined molecules, such as tumor necrosis factor (TNFα, reviewed in Le and Vilcek, 1987), prostaglandin E_2 (PGE_2; Williams, 1979), or immune interferon (IFNγ; Zoumbos *et al.,* 1984), have been shown to inhibit hematopoiesis at other levels (see Table II-1 for summary). Although there are nonhematopoietic source cells for inhibitors such as TNF, TGFβ, and PGE_2, it is noteworthy that activated macrophages and T lymphocytes, both hematopoietic in origin, and immune cells, are potent producers of these known inhibitory molecules (reviewed in Le and Vilcek, 1987; Moses *et al.,* 1987; Broxmeyer, 1984; see Figure II-1). Therefore, it is not surprising that these cells should be the focus of research on the suppression of lymphohematopoiesis associated with postburn trauma.

Stimulators of hematopoiesis

Hematopoietic cells not only do not proliferate and mature in the absence of hematopoietic growth factors, they die (Metcalf, 1970; Tushinski *et al.,* 1982). Thus, hematopoietic growth factors influence the survival, growth, maturation and function of blood, and blood forming cells. Table II-2 lists the eight well characterized growth factors arrayed in Figure II-1. All are glycoproteins, are encoded by different genes and bind distinct cell-surface receptors at physiologic concentrations (reviewed in Metcalf, 1986). The factors fall into two major categories according to their specificity of action: lineage specific factors and hematopoietins.

All of the factors in Table II-2 have been purified, characterized, cloned and are available in recombinant form. Several recent reviews and articles detail the physiochemical properties, chromosomal location and mechanism of expression of the human factors: granulocyte colony-stimulating factor (G-CSF), colony-stimulating factor-1 (CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3; Clark and Kamen, 1987); IL-1 (Le and Vilcek, 1987), IL-4 (Paul and Ohara, 1987), and IL-6 (Ikebuchi *et al.,* 1987). All except IL-1 are encoded by single genes. IL-1 exists at least in the α and β forms, of which the α form has been documented to have effects on hematopoiesis (Stanley *et al.,* 1986; Mochizuki *et al.,* 1987). All except CSF-1 are single polypetide chains. The transcript for CSF-1 can be spliced into two messages, one encoding a 145 amino acid chain and the other a 233 amino acid chain (Wong *et al.,* 1987). Homodimers (and probably heterodimers) of either of these chains are biologically active and bind to the same receptor (Wong *et al.,* 1987). Production of most of the factors is inducible (not known yet for IL-6) with regulation at the transcriptional and mRNA turnover levels (Shaw and Kamen, 1987). Receptors to the factors have all been described (reviewed in: Metcalf, 1986; Clark and Kamen, 1987; Le and Vilcek, 1987; Ohara and Paul, 1987). Genes encoding receptors to IL-2 (Cosman *et al.,* 1984), CSF-1 (Sherr *et al.,* 1985) and IL-1 (Simm *et al.,* 1988) have been cloned. The best characterized are those of IL-2 and CSF-1, the latter of which is the product of the proto-oncogene c-*fms* (Sherr *et al.,* 1985), a transmembrane tyrosine kinase. With the exception of the CSF-1 receptor, which can be present at over 100,000 copies per cell (Guilbert and Stanley, 1986), fewer than 10,000 high-affinity receptors per cell appear to be the norm for the other factors (reviewed in Clark and Kamen, 1987).

The lineage-specific factors erythropoietin, G-CSF, and CSF-1, exert their dominant, although not exclusive, action on the production of a single blood-cell type. Erythropoietin is a classical endocrine hormone. Its production is predominantly in the kidney, serum levels correlate with the degree of hypoxia, and nothing else can replace it in stimulating the later stages of erythrocyte production (Eaves and Eaves, 1985). In contrast, G-CSF and CSF-1, although their actions within hematopoiesis are relatively limited to neutrophil and monocyte production, respectively, do not appear to be the only factors able to carry out these functions (see Figure II-1). In addition, CSF-1 appears to have an important function outside hematopoiesis on the placental trophoblast, a tissue of epithelial origin (Athanassakis, Vassialadis, Guilbert and Wegmann, submitted for publication). However, all three lineage-specific factors strongly stimulate both the proliferation of the more mature progenitor cells of their respective lineages and the function of the mature cells (reviewed in Clark and Kamen, 1987). Extrapolation places a lineage-specific factor at this level for all six lineages. The recent and very intense interest in hematopoietic growth factors should lead to the rapid characterization of growth factors, inhibitors, or conditions that uniquely specify levels of basophils, eosinophils, and platelets.

Hematopoietins are growth factors that stimulate production of several blood-cell types (Figure II-1 and Table II-2). Of these, IL-1α and IL-6 appear to act in a complementary fashion (complete synergy) on very immature progenitor cells with the other hematopoietic growth factors to stimulate production (Stanley *et al.*, 1986; Mochizuki *et al.*, 1987; Ikebuchi *et al.*, 1988). In cultures of unfractionated marrow cells, the other hematopoietins, IL-3, GM-CSF and IL-4, appear able to stimulate proliferation of both mature and immature progenitor cells (Ihle, 1985; Metcalf, 1986; Rennick *et al.*, 1987). However, as pure preparations of recombinant factors and better methods for enriching hematopoietic cells have become available, there is increasing evidence that at least two growth factors are required for complete mitogenic stimulation of individual subclasses of all hematopoietic cells (Williams *et al.*, , 1987). For example, macrophage colony formation from mouse progenitor cells in serum-free medium requires the lineage-specific factor CSF-1 plus either of the hematopoietins GM-CSF or IL-3 Guilbert, 1985).

The mechanism of hematopoietic growth factor complimentation is largely unexplored although specificity has been observed in the ability of hematopoietins to down-regulate receptors for lineage-specific factors (Walker *et al.*, 1985). Other, not entirely mutually exclusive, possibilities are that receptors for hematopoietins send qualitatively different signals than do the receptors for lineage-specific factors. However, synergy between inducible factors which are undetectable in normal biological fluids (such as IL-1, IL-3 and GM-CSF) and lineage-specific factors which are present in relatively high concentrations in normal fluids (such as erythropoietin and CSF-1) makes biological sense. Thus, immunologic induction of either macrophages or T cells would be able, with great leverage, to stimulate several hematopoietic lineages simultaneously.

Homeostasis

Normal steady-state hematopoiesis therefore represents a homeostasis between stimulation and inhibition in which inducible, short-range factors may have a minimal role. Gross immunologic perturbation resulting from bacterial invasion can be predicted to lead to rapid release of factors that both stimulate and inhibit hematopoiesis. It makes biologic sense to stimulate production of monocytes and granulocytes, but it also makes sense to protect the system from potential exhaustion by inhibiting the cycling of stem cells. At present too little of a quantitative nature is known of the balancing effects of stimulators and inhibitors on hematopoiesis to be able to effectively model responses to trauma. However, enough of a qualitative nature is known to picture such responses as complex and interactive.

Noteworthy in this regard is the plasticity of the response of a single cell, the monocyte/macrophage, to stimulation potentially arising from burn trauma. Bacterial cell wall lipopolysaccharide can induce production of tumor necrosis factor (TNF), an inhibitor of hematopoiesis, and G- and GM-CSFs, stimulators of hematopoiesis from monocytes (Warren and Ralph, 1986) Both TNF and GM-CSF can induce production of CSF-1, the growth stimulator specific to the monocyte differentiation lineage, from monocytes (Oster *et al.*, 1987; Horiguchi *et al.*, 1987). In turn, CSF-1 can induce production of TNF from monocytes (Warren and Ralph, 1986), suggesting an autocrine-amplification loop. Thus, activation of macrophage/monocytes can lead to the production of both stimulators and inhibitors of hematopoiesis and the production of such regulatory elements by monocytes is part of a highly interactive, even an autoreactive, network. The interaction of a homogeneous population of macrophages to some of the elements of this network is examined below.

REGULATION OF MOUSE MACROPHAGE PROLIFERATION

Individual subclasses of progenitor cells constitute less than 0.1% of total marrow cells, and thus cannot be readily be studied in isolation or in number. We have found that mouse bone marrow-derived macrophages (BMM), which can be obtained as relatively homogeneous populations, respond to the same growth factors as macrophage progenitors; i.e., CSF-1, GM-CSF and IL-3 (Guilbert, 1985). However, unlike the progenitors, which required two factors for proliferation, BMM responded to each factor alone, albeit to differing degrees. We therefore asked whether the above growth factors cooperatively stimulated BMM proliferation. Preliminary studies indicated only marginal cooperation between the factors.

Reasoning that perhaps larger cooperative effects might be masked by the heterogeneity of primary cells, we have reexamined the question using a repeatedly cloned, growth-factor dependent cell line, S1, derived from BMM as previously described (Branch and Guilbert, 1986). Like BMM, S1 cells come rapidly to quiescence in the absence of growth factors and proliferate to differing degrees in response to maximally stimulating levels of the three macrophage growth factors (Table II-3). However, unlike BMM, these cells respond to combinations of CSF-1 and the hematopoietins synergistically (Figure II-2). In contrast, combinations of GM-CSF and IL-3 appear unable to act synergistically. The ability of low levels of CSF-1 to enhance the mitogenic effects of GM-CSF is illustrated in Figure II-3. Synergy between IL-1 and CSF-1 is also observed in a similar experiment (Figure II-4). Surprisingly, G-CSF, a factor assigned specificity to the neutrophil lineage (Walker *et al.*, 1985), appears slightly inhibitory for S1 cell proliferation stimulated by CSF-1 (Figure II-2).

These results indicate that CSF-1 synergistically cooperates with GM-CSF, IL-3 and IL-1 to stimulate the growth of mouse macrophages and may negatively cooperates with G-CSF. The observation that GM-CSF and IL-3 do not act synergistically indicates certain specificity in the complementation. It is of interest that CSF-1, GM-CSF, IL-1 and G-CSF, but not IL-3, are macrophage products subject to induction (see Figure II-1). These observations suggest that macrophages, like other hematopoietic cells, require at least two growth factors for full mitogenic stimulation, and that one factor is supplied by the target cell in response to the other. The results also indicate that CSF-1-induced release of cytokines such as G-CSF may inhibit CSF-1 stimulated proliferation (also see Appendix 1a). How much autocrine synergy contributes to the proliferation stimulated by CSF-1, GM-CSF and IL-3, and autoinhibition modulates that stimulation, remains to be determined.

Table II-1

Soluble factors inhibiting hematopoiesis

Factor	Progenitor target[a]	Source[b]	Range
PGE_2	Late[c]	Monocytes	Short/long
IFNα/β	Intermediate	Ex/monocytes	Short
IFNγ	Intermediate	T cells	Short
TNFα	Intermediate	Monocytes/T cells	Short
TNFβ	Intermediate	T cells	Short
TGFβ	Early	Ex/monocytes/T cells	Short

[a] Maturity of hematopoietic progenitor cells in cell lineages, referring to Figure II-1: late, CFU-E, CFU-GM, etc., or more mature; early, CFU-GEMM or less mature; intermediate, progenitors between early and late.

[b] Ex, Outside lymphohematopoiesis

[c] Monocyte progenitors are especially sensitive

IFN, Interferon; TNF, tumor necrosis factor; TGF, transforming growth factor; CFU, colony-forming unit, where -E, erythroid; -GM, granulocyte, macrophage; -GEMM, granulocyte, erythroid, macrophage, megakaryocyte.

Table II-2

Soluble factors stimulating hematopoiesis

Factor	Target	Source[a]	Range
Erythropoietin	Erythroid[b]	Ex/Monocytes	Long
G-CSF	Neutrophil[b]	Ex/Monocytes	Short
CSF-1	Monocytic[b]	Ex/Monocytes	Long/Short
GM-CSF	Hematopoietic	Ex/Monocytes/T Cells	Short
IL-1	Hematopoietic	Ex/Monocytes/T Cells	Long/Short
IL-2	Hematopoietic	T Cells	Short
IL-3	Hematopoietic	T Cells	Short
IL-4	Hematopoietic	T Cells	Short
IL-6	Hematopoietic	Ex/Monocytes	?

[a] Ex, Outside lymphohematopoiesis.

[b] Differentiation lineage specific.

Table II-3

Stimulation of S1 cell proliferation by CSF-1, GM-CSF and IL-3

Colony-stimulating Factor	Maximal response[a] (cpm)
None	955 ± 420
CSF-1	31,844 ± 8,162
GM-CSF	37,426 ± 2,029
IL-3	5,158 ± 653

[a] Proliferation assessed as uptake of ^{3}H-TdR as described in the legend to Figure II-2. Levels of growth factors are maximally stimulatory for S1 cells: CSF-1 = 5 x 10^3 U/mL; rGM-CSF = 250 ng/mL; IL-3 = 1000 U/mL.

Figure II-1

Regulation of hematopoiesis by hematopoietic growth factors

Scheme modified from Clark and Kamen (1987). Progenitor cells denoted in *large letters*, factors in *small letters*. Factor production by macrophages and T lymphocytes denoted in *boldface*.

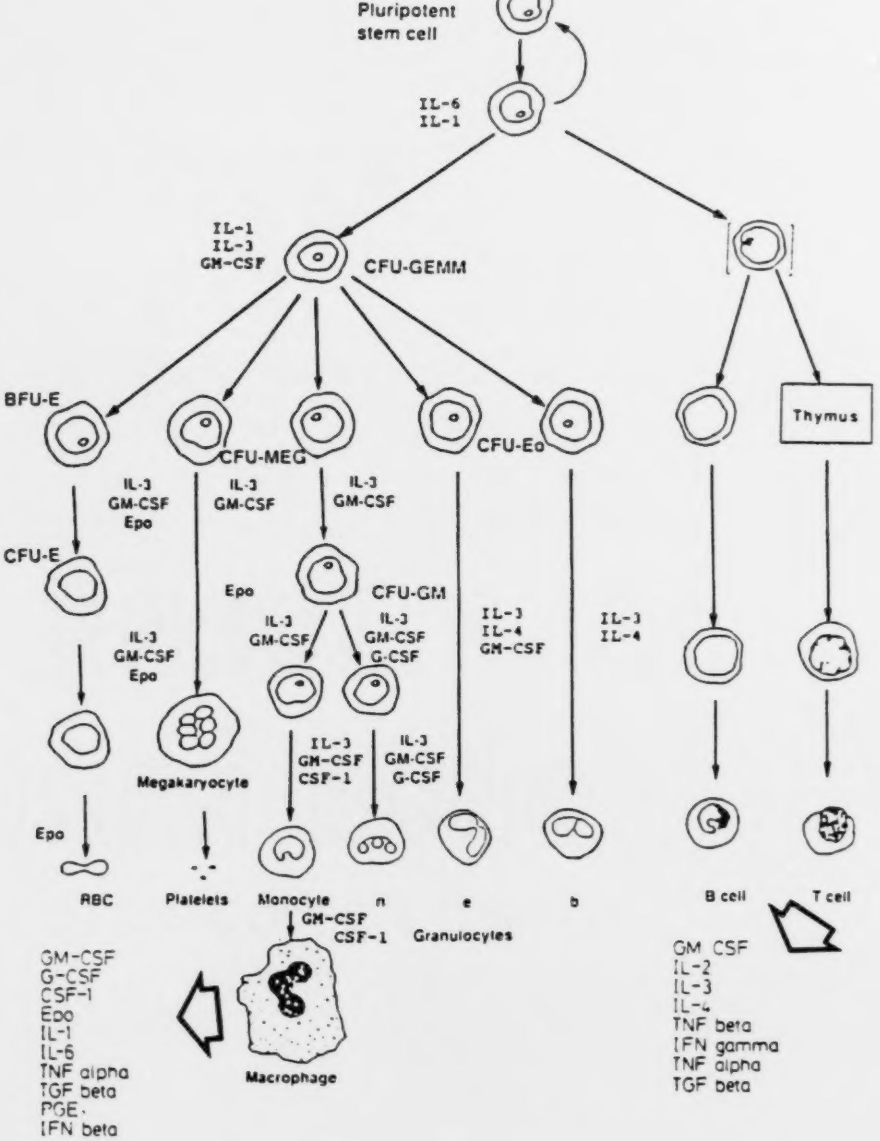
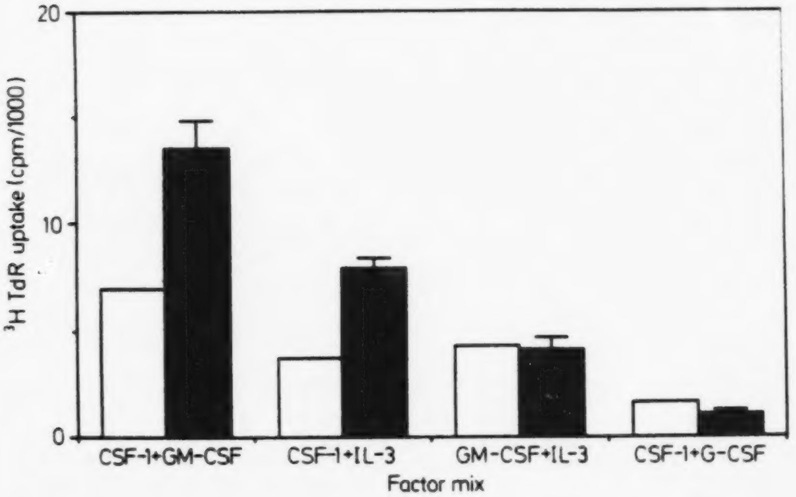

Pluripotent stem cell
IL-6
IL-1
IL-1
IL-3
GM-CSF
CFU-GEMM
BFU-E
CFU-MEG
CFU-Eo
Thymus
IL-3
GM-CSF
Epo
IL-3
GM-CSF
IL-3
GM-CSF
CFU-E
Epo
CFU-GM
IL-3
GM-CSF
Epo
IL-3
GM-CSF
IL-3
GM-CSF
G-CSF
IL-3
IL-4
GM-CSF
IL-3
IL-4
Megakaryocyte
IL-3
GM-CSF
CSF-1
IL-3
GM-CSF
G-CSF
Epo
RBC
Platelets
Monocyte
n
e
b
Granulocytes
B cell
T cell
GM-CSF
CSF-1
Macrophage
GM-CSF
G-CSF
CSF-1
Epo
IL-1
IL-6
TNF alpha
TGF beta
PGE2
IFN beta
GM CSF
IL-2
IL-3
IL-4
TNF beta
IFN gamma
TNF alpha
TGF beta

Figure II-2

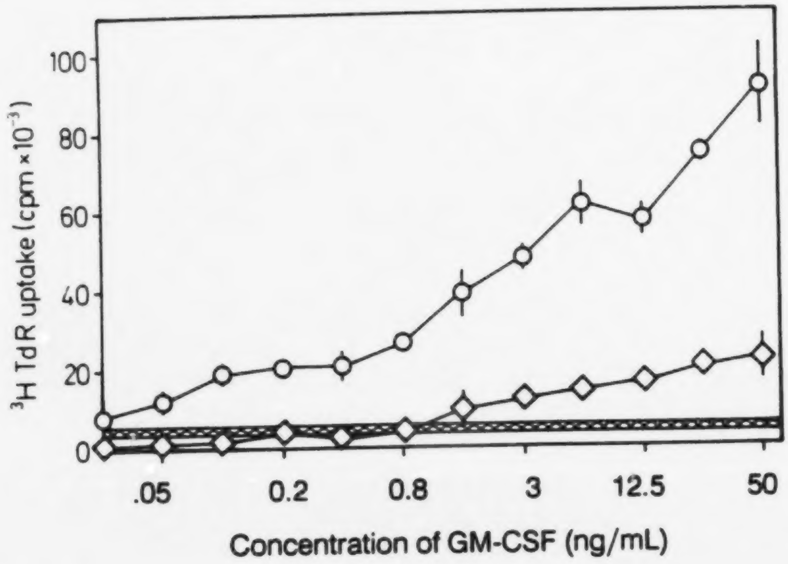
Synergistic stimulation of mouse macrophage proliferation



Synergistic stimulation of mouse macrophage proliferation by CSF-1, GM-CSF, IL-3 and G-CSF. 1000 cells per well were cultured in 96 well dishes with the combinations of factors in 100 μL 15% fetal bovine serum in Iscove's modified Dulbecco's medium for 3 days, then pulsed for 4 hr with 1 μCi ^{3}H-TdR before harvesting. CSF-1 (pure natural, 3000 U/mL), GM-CSF (pure recombinant, 250 ng/mL), IL-3 (pure natural, 1000 U/mL) were used at levels which maximally stimulated S1-cell proliferation when used alone. G-CSF (pure recombinant) was added at 1000 U/mL. *Unshaded*, predicted (Σ means of individual stimulations + Σ SD); *shaded*, observed.

Figure II-3

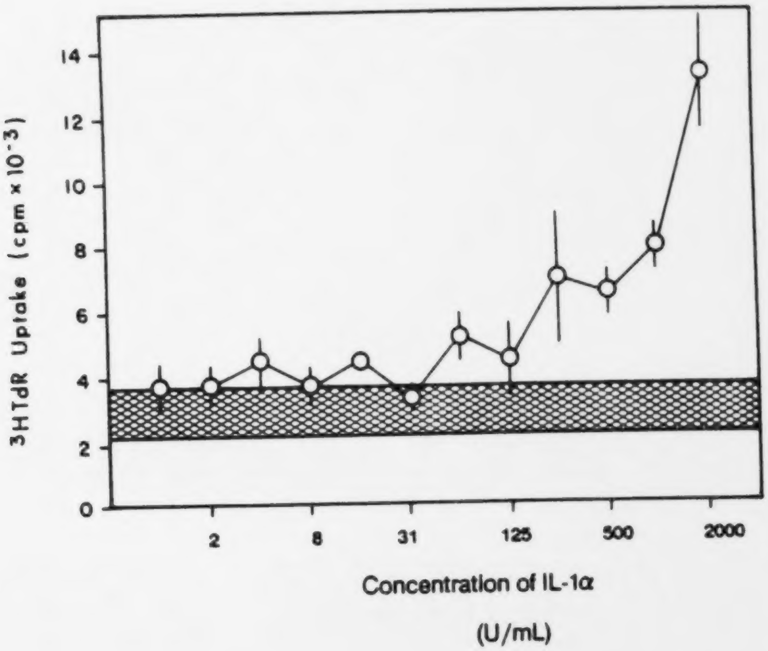
Stimulation of S1 cell growth by GM-CSF



Stimulation of S1 cell growth by GM-CSF with (○) and without (◇) suboptimal concentrations of CSF-1 (200 U/mL). For materials and methods, see legend Figure II-2. *Hatched area* depicts uptake stimulated by CSF-1 alone ± SD. *Abscissa,* doubling dilutions of GM-CSF in the cultures expressed as $\log_2$ dilution of a stock containing 100 ng/mL.

Figure II-4

Stimulation of S1 cell growth by IL-1α



Stimulation of S1 cell growth by IL-1α with (○) and without (—) suboptimal levels of CSF-1 (500 U/mL). For materials and methods, see legend Figure II-2. *Hatched area* depicts ^{3}H-TdR uptake stimulated by CSF-1 alone ± SD. *Abscissa*, doubling dilutions of IL-1α (pure recombinant) in the cultures expressed as $\log_2$ dilution of a stock containing 4000 U/mL.

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CHAPTER III

SYNERGISTIC STIMULATION OF MACROPHAGE PROLIFERATION BY THE CYTOKINES TUMOR NECROSIS FACTOR-ALPHA AND COLONY-STIMULATING FACTOR-1[1]

OVERVIEW

This chapter addresses specific objective 2 of the thesis, the isolation and characterization of a highly homogeneous, bone marrow-derived, primary macrophage cell population, closely matched to a growth factor-dependent macrophage cell line. Also, specific objective 3 is addressed, the examination of the relative roles for various lympho-hematopoietic cytokines in the proliferation of mouse macrophages. This chapter presents the original observation that TNFα can cooperate, in a synergistic manner, with maximally stimulatory levels of CSF-1, but not GM-CSF, to increase further the proliferation of both BMM and the S1 macrophage cell line (see Table III-2).

This chapter indicates the close similarities between the growth factor-dependent macrophage cell line S1 and culture-selected primary BMM (see Table III-1). Examination of fifteen cytokines indicated that only CSF-1, GM-CSF or IL-3 stimulated macrophage proliferation on their own (see Chapter II and Appendix 1). However, of the cytokines and agents examined, some appeared to down-modulate the proliferative response to CSF-1, including G-CSF (see Chapter II and Appendix 1a), GM-CSF (see Appendix 1b and 1c), IL-6, IL-4, TGFβ, LPS, IFNγ and PGE_2 (see Appendix 1d, 1i, and 1j). In contrast, only IFNγ and PGE$_2$ appeared to inhibit the GM-CSF response (see Appendix 1e and 1i). Examination of cooperative interactions of various cytokines on the CSF-1 or GM-CSF proliferative responses showed only two which could positively modulate the proliferative response of normal BMM. These were TNFα and the related cytokine TNFβ (see Table III-2; Appendix 1g and 1j). These cytokines showed specificity in that they would synergize with CSF-1 but not with GM-CSF. IL-1α, which shares many of the biological properties of TNFα, did not augment the CSF-1 response of normal BMM (see Appendix 1f). The results document a possible paracrine role for TNFα acting directly on macrophages to provide a cooperative signal with the macrophage growth factor CSF-1. These results are pivotal to the thesis and argue that CSF-1 does not send an optimal mitogenic signal, that mouse macrophages require two signals (CSF-1 and TNFα) for full mitogenic responsiveness.

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[1] A version of this chapter has been published. Branch, D.R., Turner, A.R. and Guilbert, L. J. (1989) Synergistic Stimulation of Macrophage Proliferation by the Monokines Tumor Necrosis Factor-Alpha and Colony-Stimulating Factor-1. Blood 73:307-311.

INTRODUCTION

Monocytes and macrophages synthesize a number of regulatory proteins (cytokines) that play important roles in a variety of cellular functions (1). Among these cytokines are tumor necrosis factor-α (TNF), originally described for its cytostatic and cytocidal effects on neoplastic cells (2), and CSF-1, the macrophage-specific, colony-stimulating factor (3). Both these factors have been well characterized, purified to homogeneity, and molecularly cloned (2-7). CSF-1 strongly promotes mononuclear phagocyte survival, function, proliferation, and differentiation (3). TNF acts beyond the original definition and has been shown to exert multifunctional effects on normal cells, including hematopoietic cells (8). It inhibits colony formation stimulated by CSFs (9,10), induces, in cooperation with gamma interferon, monocytic differentiation of the human myelomonocytic cell line HL-60 (11), and stimulates production of CSF-1 from human monocytes (12). Conversely, CSF-1 can induce production of TNF from monocytes (13).

The above observations indicated that although the actions of TNF and CSF-1 exert different effects on hematopoietic cells, they may be linked via reciprocally induced production by monocytes/macrophages. Whether the two cytokines also modulate each other's actions on macrophages is unknown. We have therefore asked in this report whether TNF modifies in any way the growth-stimulating effects of CSF-1 on mouse bone marrow-derived macrophages (BMM). We find that although TNF has very little mitogenic activity alone, it almost doubles the maximal growth stimulation CSF-1 exerts on BMM. Furthermore, it rapidly and transiently down-regulates cell-surface expression of the CSF-1 receptor on the same cells.

MATERIALS AND METHODS

Cells

BMMs were prepared from the femurs of C3H/HeJ (H-2^k) mice as previously described (14), with modification to allow harvest of adherent cells at day 7 of culture. Bone marrow cells were cultured in tissue culture dishes at 10^6/mL in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) supplemented with 15% (vol/vol) fetal bovine serum (FBS, Flow Laboratories, McLean, VA), 10% (vol/vol) medium conditioned by the myelomonocytic cell line WEHI-3 (American Type Culture Collection, Rockville, MD) and 2000 U/mL stage 1 mouse CSF-1 (15). After 24 hours, nonadherent cells were transferred to new tissue culture dishes with fresh growth medium and cultured for an additional 2 days, at which time nonadherent cells (d3BMMs) were harvested and cryopreserved in liquid nitrogen. When required, frozen aliquots of d3BMMs were thawed and plated into culture in **Petri plastic** dishes with IMDM containing FBS (15% vol/vol) and stage 1 CSF-1 (3000 μ/mL). After 2 days of culture, the nonadherent cells were harvested and replated into new Petri plastic dishes with fresh CSF-1 (3000 U/mL)-containing growth medium. After 2 additional days in culture, adherent cells (d7BMM) were harvested following 5 min treatment at 4°C with 5mM ethylenediamine tetraacetic acid (EDTA) in phosphate buffered saline (PBS).

The growth factor-responsive mouse macrophage cell line 5/10.14 was isolated from *v-myc*-transformed C3H/HeJ bone marrow cells in our laboratory as previously described (16). The S1 line was cloned from 5/10.14 by limiting dilution culture with high concentrations of CSF-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and was selected for low autonomous growth and high responsiveness to CSF-1, GM-CSF, and IL-3. Early passage aliquots are cryopreserved and, prior to any experiment, recultured for one to 3 days in Petri dishes containing IMDM supplemented with 15% FBS, 20% L-cell conditioned medium, and 10-20 ng/mL recombinant GM-CSF.

Cell surface marker analysis and morphology

The rat monoclonal antibody (mAb) F4/80 was used as unpurified culture supernatant of the F4/80 hybridoma obtained from Dr. S. Gordon (17). Monoclonal rat antibody to the macrophage C3bi receptor (MAC-1) was used as supernatant from the original hybridoma (clone M1/70.15.11.5, American Type Culture Collection). Murine monoclonal anti-H-2K^k (IgG2a, clone 16-3-1N, ref. 18) and anti-H-2D^d (IgG2a, clone 34-5-8s, ref. 19) were used as unpurified ascites. Murine monoclonal anti-I-A^k (Class II, IgG2a, clone 10-3.6.2, ref. 20) and anti-I-A^d (IgG2a, clone MKD6, ref. 21) were used as purified ascites. Murine monoclonal IgM anti-Thy-1.2 was purchased from New England Nuclear, Boston, MA. Murine monoclonal anti-Ly2.1 (IgG2a, clone 116-13.1) and rat monoclonal anti-L3T4 (clone GK1.5) were used as ammonium sulfate purified culture supernatant from the original hybridoma (American Type Culture Collection). Murine monoclonal IgM anti-asialo-GM$_1$ (clone 49H8, ref. 22) was used as affinity-purified ascite. Cell-surface antigen expression was assessed by indirect immunofluorescence using fluorescein (FITC)-conjugated affinity purified F(ab')$_2$ fragment goat anti-mouse Ig and goat anti-rat IgG (Cappel Laboratories, Cooper Biomedical, Malvern, PA) and analyzed on a Coulter EPICS V flow cytometer. Cells were pre-incubated for 30 minutes at 4°C with undiluted normal goat serum. The serum was removed and the cells resuspended in diluent PBS containing 10% (vol/vol) normal human AB serum and 2% (vol/vol) FBS. An equal volume of mAb was added to cells, incubated for 1 hour at 4°C, and washed twice with PBS. One-hundred μL of the appropriate FITC-conjugated second antibodies were added for another hour's incubation at 4°C. The cells were washed twice with PBS and resuspended in PBS containing 1% (vol/vol) formaldehyde prior to fluorescence analysis. Background immunofluorescence was determined using PBS. Negative and isotype controls were compared with background levels. Phagocytosis was assessed by uptake of FITC-impregnated latex beads (Polysciences, Warrington, PA) after incubation for 1 hour at 37°C. Cytospin preparations of cells from log-phase growing cultures were prepared and stained with Wright-Giemsa before differential analyses. A minimum of 200 cells were counted and observed for characteristic morphologic features.

Cytokines

Pure recombinant human TNF (10^8 U/mg) was purchased from Amgen Biologicals, Thousand Oaks, CA, stored at 2-8°C and used within 2 months of arrival. Highly purified, natural mouse CSF-1 (23) was a gift from Dr. E.R. Stanley, New York, NY.

Proliferation assays

Proliferation of macrophages in 2 day cultures was monitored by uptake of radioactive tritiated-thymidine (^{3}H-TdR, New England Nuclear, Boston, MA, 20 Ci/mmol, 1 μCi/microwell) in the last 4 hours of culture using a previously described 96-well microplate method (16). Cytokines were diluted in IMDM containing 15% (vol/vol) FBS to give a final volume of 50 μL/well. Cells for proliferation assays were harvested from Petri dishes as described above and washed four times with sterile PBS, and 10^3 cells in 50 μL were added to all wells of the microtiter plate. The plates were incubated at 37°C in an atmosphere of 5% CO_2 in air for 2 days, and then ^{3}H-TdR was added for 4 hours. After the strongly adherent macrophages were loosened with 0.05% Triton X-100 for 5 to 10 min., the cells were harvested using a Titertek 550 cell harvester (Flow Laboratories) and ^{3}H-TdR incorporation was measured on a scintillation counter.

^{125}I-CSF-1 binding assays

CSF-1 was radio-iodinated with Chloramine T as previously described to approximately 3 x 10^{18} cpm/mol (15). The biologic activity of each iodinated preparation was determined by a proliferation assay using a previously described colorimetric (MTT) assay (16). Binding of ^{125}I-CSF-1 was performed at 4°C as previously described (14), with modification to accommodate analysis in 96-well microtiter dishes. Log-phase growing cells were harvested and washed 4 times with PBS, resuspended in IMDM containing 15% (vol/vol) FBS and aliquoted at 2 x 10^4 per well per 50 μL. After overnight incubation in the absence of CSF-1 to up-regulate CSF-1 receptors (14), TNF was added to selected wells at a final concentration of 1600 ng/mL for 1, 2, 4, and 24 hours. Each plate was washed twice with 200 μL/well ice-cold PBS; 50 μL of IMB (IMDM lacking bicarbonate and adjusted to 280 mOsm and pH 7.35, ref. 14) containing 10% FBS (vol/vol) was added to each well; and the plate was cooled on ice for 20 min. After cooling, 10 μL of ^{125}I-CSF-1 (50,000 cpm) was added to each well, the solutions quickly mixed on a shaker, and the reaction allowed to proceed for 2 hours on ice. Nonspecific binding was determined by preincubating the cells for 2 hours with a saturating level (2 nM) of nonradioactive CSF-1 before addition of ^{125}I-CSF-1 (15). The binding reaction was stopped by washing 6 times with 200 μL ice-cold PBS. Cell-associated ^{125}I and DNA were quantitatively recovered from individual microwells following a 30-min incubation with 100 μL 0.1M NaOH at 60°C. The contents of each well were combined with washes of 100 μL of 0.1M Tris (hydroxymethyl) aminomethane (TRIS) containing 0.1N HCl (final pH 8.0) and 100 μL of 5M NaCl. Samples were counted for ^{125}I on a gamma counter, and then the DNA content of individual samples was quantitated fluorimetrically on a TKO 100 mini-fluorometer (Hoefer Scientific, San Francisco, CA) after reaction with bis-benzimidazole (Hoechst 33258, ref. 24, Sigma Chemical, St. Louis, MO). The DNA content of all microwells containing either BMM or S1 cells was the same (36 ± 3 ng per 2 x 10^4 cells); thus, the binding results are given in terms of cpm per 2 x 10^4 cells added.

RESULTS

Characterization of macrophage populations

The primary d7BMM and the S1 cell line were comparatively characterized for homogeneity, morphology, and expression of cell-surface markers that typify macrophages (Table III-1). Both populations are adherent and phagocytic to the same degree and express similar levels of cell-surface markers indicative of relatively mature macrophages (e.g. F4/80, MAC-1, class II). Morphologically, both populations also show similar features (e.g., single nucleus, intermediate nucleus-to-cytoplasm ratio, slightly basophilic and vacuolated cytoplasm) typical of monocytes/immature macrophages. Therefore, within the resolution of this analysis, primary d7BMM are almost indistinguishable from the cloned S1 macrophage cell line.

Stimulation of macrophage proliferation by TNF and CSF-1

CSF-1, when added at maximally stimulating (plateau) or at suboptimal levels, stimulates the proliferation of both macrophage populations (Table III-2). TNF alone has almost no effect on the proliferation of mouse macrophages although in 9 independent experiments using high concentrations ($\geq$ 1250 ng/mL), ^{3}H-TdR uptake was invariably above control levels. In contrast to its marginal effect alone, TNF more than doubles the maximal ability of CSF-1 to stimulate d7BMM proliferation (Table III-2). Indeed, at levels of less than 200 ng/mL, TNF does not significantly stimulate macrophage proliferation but is still able to strongly enhance (> 2-fold) CSF-1 stimulation of proliferation. The same results (synergy between TNF and CSF-1) were obtained in 8 independent experiments using ^{3}H-TdR uptake as an index of growth and in yet another experiment in which MTT reduction (16) was used as a measure of viable cell number (data not shown). The fact that this action of TNF is not mediated by a non-macrophage accessory cell in the primary population is shown by similar effects on the S1 cell line.

Effect of TNF on binding of CSF-1 to macrophages

Comparison of binding assays performed under the same conditions (^{125}I-CSF-1 concentration, cell number, temperature, and time of reaction) shows S1 cells to have approximately one-third the number of specific CSF-1 binding sites that d7BMM have (Table III-3). Incubation of both populations with TNF at 37°C caused rapid and almost complete (87% - 94%) loss of CSF-1 binding. The down-modulation of binding sites by TNF was transient, with specific binding reaching its minimum at 1 hour and returning to normal (d7BMM) or greater (S1) values by 24 hr (Table III-3). This transience was not due to loss of TNF in culture, because significant levels of TNF remain even after 3 days of culture (data not shown, determined by lysis assay using TNF-sensitive mouse L-929 cells, ref. 5, 25).

DISCUSSION

In contrast to previous reports of antiproliferative effects of TNF on immature hematopoietic cells (9, 10), this report documents a novel, growth-stimulating action on more mature macrophages. TNF alone appears to be marginally mitogenic. However, this effect could well be due to its induction of CSF-1 release, which occurs with both primary d7BMM and the S1 macrophage cell line (data not shown), and which others have reported with human monocytes (12). The major stimulatory effect of TNF is in cooperation with CSF-1, hitherto regarded as the most potent stimulator of mouse macrophage proliferation (3, 26). The synergistic stimulation of growth by CSF-1 and TNF is observed with primary cells and a cloned cell line, both of C3H/HeJ origin; thus, the effects of TNF are not mediated by bacterial lipopolysaccharide (LPS) contaminants, are exerted on the same cells that bear the CSF-1 receptor, and are not limited to cell lines. Indeed, since the macrophage is the major TNF-producing cell (2, 4, 5, 25), cooperation between TNF and CSF-1, the macrophage-specific growth factor, would most likely occur *in vivo*.

TNF may enhance the mitogenic action of CSF-1 by mimicking, at greater amplitude, a part of the usual pleiotropic response to the growth factor. One action that TNF mimics at greater amplitude is down-modulation of CSF-1 binding (Table III-3). CSF-1 only partially down-regulates its own receptor on d7BMM (14), whereas TNF rapidly and almost completely does so. This down-modulation of CSF-1 binding by TNF is not due to receptor cross-reactivity, because TNF does not compete with ^{125}I-CSF-1 for binding to its receptor at 4°C (data not shown). The correlation between transient and complete down-modulation of CSF-1 receptor and synergistic growth stimulation of macrophages has been observed before with tumor-promoting phorbol diesters (27, 28) which are direct activators of protein kinase C (29), and with other hematopoietic growth factors (30, 31). These results therefore indicate that receptor down-modulation is either important to the mitogenic action of CSF-1 or reflects an important response.

Alternatively, TNF may exert an effect on macrophages that CSF-1 cannot; i.e., it may complement CSF-1 to stimulate growth. Since CSF-1 has been reported to induce TNF production from mononuclear phagocytes (13), no mitogenic response to CSF-1 may be free of TNF effects. Thus, the possibility exists that the macrophage, like its progenitors (26, 32), may require other growth factors in addition to CSF-1 for proliferation, and that TNF may be one of these requirements.

Table III-1

Cell surface markers and morphological findings[*]

Property	Percent Positive	
	d7BMM	S1 Cell Line
Adherence	>95	>95
Phagocytosis	>98	>98
Cell Surface Markers:		
Background Controls[#]	10	18
F4/80	82	97
MAC-1	92	91
Class I ($H\text{-}2^K$)	91	94
Class II (Ia^k)	39	48
Morphology:		
Immature Macrophages (single nucleus)	92	90
Immature Macrophages (two nuclei)	1	6
Other (monocytoid)	7	4

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[*] Properties determined as described in Materials & Methods.

[#] Background results (± 5%) were obtained for PBS, $H\text{-}2^D$, Ia^d, Thy-1.2, Ly2.1, L3T4, and asialo-GM_1

Table III-2

Stimulation of macrophage proliferation by CSF-1 and TNF alone and in combination

	Proliferation Response*			
	Maximum Levels├		Suboptimal Levels╞	
Stimulus	Calculated§ Additive	Observed	Calculated Additive	Observed
d7BMM╟				
None		396 ± 132		337 ± 52
TNF		591 ± 135		300 ± 145
CSF-1		4,852 ± 344		3,134 ± 257
TNF + CSF-1	5,443 ± 479	10,424 ± 386	3,434 ± 402	6,639 ± 387
S1 Cell Line╟				
None		102 ± 46		122 ± 42
TNF		1,115 ± 380		184 ± 53
CSF-1		24,925 ± 1,491		7,885 ± 620
TNF + CSF-1	26,040 ± 1,871	41,973 ± 2,809	8,069 ± 673	22,363 ± 449

* Cpm ± SD of ^{3}H-TdR Uptake.

├ Maximum stimulatory (plateau) levels when used alone: TNF = 1,250 ng/mL; CSF-1 = 2.5 x 10^3 U/mL.

╞ Suboptimal stimulatory levels: TNF = 125 ng/mL; CSF-1 = 25 U/mL (d7BMM) and 100 U/mL (S1 cell line).

§ Σ means ± Σ SD.

╟ 10^3 cells per well, cultured for two days (see Materials & Methods section).

Table III-3

Effect of TNF on ^{125}I-CSF-1 binding at 4°C[#]

Conditions	^{125}I-CSF-1 Binding[*] d7BMM	^{125}I-CSF-1 Binding[*] S1 Cell Line
Nonspecific Binding	67 ± 10	71 ± 12
Medium Alone	3,168 ± 269	1,149 ± 139
TNF for 1 hour	492 ± 43	178 ± 25
TNF for 2 hours	1,178 ± 45	365 ± 37
TNF for 4 hours	2,195 ± 212	746 ± 18
TNF for 24 hours	3,648 ± 497	4,035 ± 327

[#] See Materials & Methods.

[*] Counts per minute per 2 x 10^4 cells; assays done in triplicate.

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CHAPTER IV

A SPECIFIC AND RELIABLE BIOASSAY FOR THE DETECTION OF FEMTOMOLAR LEVELS OF HUMAN AND MOUSE TUMOR NECROSIS FACTORS[1]

OVERVIEW

During the course of the thesis research, it became apparent that TNFα production from macrophages of C3H/HeJ origin could not be detected with available bioassays. Thus, either these macrophages were unable to produce TNFα or the TNF bioassays were not sensitive enough to detect very low levels of TNFα. I decided that the latter explanation needed to be addressed. Therefore I developed a bioassay that could detect femtomolar levels of TNFs. This bioassay is presented in this chapter. This bioassay is approximately 180-fold more sensitive than other commonly used bioassays for the detection of human TNFα, and is able to measure murine-derived TNFα down to 200 fg/mL (see Figure IV-7). This bioassay was essential for determining whether C3H/HeJ or C3H/HeN macrophages could produce bioactive TNFα in response to macrophage growth factors. The bioassay was used in addressing the specific objective under Hypothesis II of the thesis: To determine whether TNFα acts as an endogenous autocrine stimulatory molecule

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[1] A version of this chapter has been published. Branch, D.R., Shah, A. and Guilbert, L.J. (1991) A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. Journal of Immunological Methods 143:251-261.

INTRODUCTION

TNFα, first discovered in the sera of mice primed with BCG and subsequently injected with endotoxin, was named for its ability to cause hemorrhage and necrosis of transplanted murine tumors (Carswell *et al.*, 1975). TNFβ (also known as lymphotoxin) was originally characterized as a cytotoxic factor produced by T cells (Granger and Williams, 1968). Cells of the mononuclear phagocyte lineage are the primary source of TNFα although other cell types, including lymphocytes and mast cells, can also produce this protein (Semenzato, 1990). TNFβ is produced only by lymphocytes, in the murine, primarily helper Type 1 T-cells (Mosmann and Coffman, 1989; Semenzato, 1990).

Both TNFα and TNFβ have been characterized, cloned and expressed, and are available as recombinant proteins (Pennica *et al.*, 1984; Pennica *et al.*, 1985; Gray *et al.*, 1984). Although they share only 30% homology at the DNA level, these proteins bind to the same receptor and have similar biological effects, including modulation of properties of vascular endothelium, induction of other cytokines, induction of antiviral activity, and stimulation of bone resorption, angiogenesis, fibroblast growth and tumoricidal activity (reviewed in Beutler, 1990 and Semenzato, 1990). TNFα appears to be a major mediator of inflammatory responses and over production results in the extensive tissue damage associated with endotoxemia and cachexia (Beutler and Cerami, 1988).

Since these molecules play major roles in biological responses and can contribute to pathological states, an *in vitro* assay for their detection and quantification is desirable. A number of RIA, ELISA and bioassays presently exist (reviewed in Meager *et al.*, 1989). However, the lack of sensitivity and/or stability and reproducibility of currently available bioassays, and the lack of assurance that only biologically relevant TNF is being detected with ELISA or RIA methods, precludes definitive testing for TNF levels in many *in vitro* culture systems or *in vivo* in animal models or humans (Beutler, 1990).

In this paper, we describe an improved bioassay that addresses many of these problems. We have isolated a stable TNF sensitive subclone of the fibroblastoid cell line L929 (L929-8) and have optimized a cytolytic bioassay, to detection limits in the femtomolar range, even in the presence of high serum concentrations. The optimized assay is simple, highly reproducible, detects both rodent and human TNFs and appears specific only for the TNFs.

MATERIALS AND METHODS

Cell lines

The murine fibroblastoid cell line L929 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture using Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, VA) containing 15% (vol/vol) fetal bovine serum (FBS, Gibco). The murine fibrosarcoma cell line WEHI-164 clone 13 (Espevik and Nissen-Meyer, 1986) was obtained from Dr. T. Mosmann (University of Alberta) and maintained in culture using RPMI-1640 (Gibco) containing 10% (vol/vol) FBS plus 10^{-4}M 2-mercaptoethanol.

Cytokines and chemicals

E. *coli* derived murine recombinant TNFα (1.2 x 10^7 U/mg; Pennica *et al.*, 1985) was provided by Genentech (So. San Francisco, CA). Human E. *coli* recombinant TNFβ (Genentech; 2.1 x 10^8 U/mg; Gray *et al.*, 1984) was a gift of Dr. A. Rabinovitch (University of Alberta). Human recombinant TNFα was a gift of Hofmann La Roche (Basel, Switzerland). Additional cytokines were used during specificity testing and included IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, GM-CSF, G-CSF, CSF-1, Epo, LIF, TGFβ, IFNγ and NGF (see Table IV-3). Lipopolysaccharide (LPS from E. *coli*, strain 026-B6), prostaglandin E_2 (PGE_2) and dexamethasone were purchased from Sigma Chemical, St. Louis MO. Cyclosporin A was purchased from Sandoz Laboratories, Basel, Switzerland. Cytokines and chemicals were diluted in IMDM containing 15% (vol/vol) FBS prior to use.

TNF bioassays using L929 cells

Bioassay using mitomycin C

Initially, an assay previously described by Spofford *et al.*, 1974, with minor modification (Fletcher *et al.*, 1987) was used . Briefly, 4 x 10^4 TNF sensitive L929 cells were added to wells of a 96-well flat-bottom microtiter plate in 50 μL of medium consisting of IMDM containing 5% (vol/vol) FBS plus 0.5 μg/mL mitomycin C (Sigma Chemical). After 24 hours of culture at 37°C in 5% CO_2 in air, 50 μL of recombinant human TNFα was added in triplicate or quadruplicate to the wells, the plates cultured a further 48 hours, then pulsed for 4 hours with a 10% solution of the tetrazolium dye MTT (Mosmann, 1983). The resulting blue reduction product was dissolved in isopropanol made 0.04N in HCl and the optical density determined spectrophotometrically (reference wavelength = 630 nm; sample wavelength = 570 nm) as previously described (Branch and Guilbert, 1986).

Bioassay using actinomycin D

The above procedure was further modified by replacing mitomycin C with actinomycin D (AMD; Ostrove and Gifford, 1979; Kramer and Carver, 1986) as follows: Cells were added to microtiter wells to a final concentration of 5 x 10^4 cells per well in 50 μL of IMDM containing 5% (vol/vol) FBS plus 2 μg/mL AMD (Boehringer Mannheim, Laval, Quebec). The microtiter plates were incubated at 37°C in 5% CO_2 in air for one to two hours, 50 μL of recombinant TNFα sample added, and the plate incubated for an additional 16 hours prior to addition of MTT and reading as above.

TNF bioassay using WEHI-164 clone 13

This assay was carried out as previously described (Espevik and Nissen-Meyer, 1986). Briefly, 50 μL of WEHI-164 clone 13 cells, without AMD, were added to microwells in a 96-well flat-bottom microtiter plate (2 x 10^4 cells/well).

Different dilutions of recombinant human TNFα were added to the target cells. The plates were incubated at 37°C in the presence of 5% CO_2 in air for 20 hours and the percentage of viable cells determined by MTT reduction as described above (Mosmann, 1983; Branch and Guilbert, 1986).

Controls and calculations

Controls for growth inhibition assays consisted of medium without TNF, which was the 100% viable cell control (negative control), and distilled water or saturating concentrations of TNF to give maximal cell killing. The sensitivity of target cell populations to the effects of TNF was expressed as the percent inhibition of cell growth (percentage of non-viable cells) calculated using the following formula:

$$\text{Percent Inhibition of cell growth} = [(OD_C - OD_X) / (OD_C - OD_0)] \times 100$$

where OD_C = optical density of 100% viable cell control.
OD_0 = optical density of 0% viable cell control
OD_X = optical density of culture containing TNF.

Results are presented as the mean of triplicate or quadruplicate determinations. Standard deviations (SD) generally were $\leq 15\%$ of the mean. In selected experiments results are presented as the mean absorbance (optical density) $\pm$ SD. The lower limit of detection was determined as the lowest concentration of TNF giving a mean optical density (OD_X) the standard deviation (SD_X) of which does not overlap with the standard deviation of the mean of the medium (no TNF) control, SD_C and OD_C, respectively: i.e.;

$$\text{Lower limit of detection} = \text{the lowest [TNF] where: } OD_C - OD_X \geq SD_C + SD_X$$

Molar concentrations were calculated by dividing the mass of TNF (in milligrams) per mL by 17,400 for rTNFα, both murine and human (Pennica *et al.*, 1984; Pennica *et al.*, 1985) and 18,600 for rhTNFβ (Gray *et al.*, 1984).

Neutral red procedure

The vital stain neutral red was used as previously described (Rich *et al.*, 1989) with minor modification. Briefly, after incubation of the target cells with TNF, 50 μL of a filtered neutral red solution [Sigma, 0.05% (wt/vol) in normal saline, stored in the dark at 4°C but warmed to room temperature prior to use], was added to each well of the microtiter plate. Plates were incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 in air, emptied by inversion, and rinsed once with 0.2 mL room temperature phosphate buffered saline (PBS) using a multichannel pipettor (Titertek, Flow Laboratories). In order to avoid variable cell loss, the rinsing was carried out quickly but carefully and the emptying by inversion of the plate as smoothly and uniformly as possible. One-hundred μL of a sodium phosphate-alcohol solution [0.05M NaH_2PO_4 in 50% (vol/vol) ethanol] was added to each well and the plate gently mixed on a shaker (Janke & Kunkel, Straufen, Germany) for at least 20 minutes. Absorbance (optical density) at 570 nm was determined using an automated microplate spectrophotometer with a reference wavelength of 630 nm as previously described for MTT (Branch and Guilbert, 1986).

RESULTS

Isolation of TNF hypersensitive cell line

The growth of the L929 cell line was only partly inhibited by TNFα when using the mitomycin C bioassay (only 50% growth inhibition at 100 ng/mL recombinant human TNFα, data not shown). Therefore, L929 cells were subcloned by limiting dilution in 96-well flat-bottom microtiter plates in 15% (vol/vol) FBS in IMDM in order to obtain a subclone more sensitive to TNF. The results in Table IV-1 on 9 subclones indicated a broad range of sensitivity to the growth inhibitory effects of 50 ng/mL recombinant human TNFα (27.8% to 93.1% inhibition of cell growth). Clone L929-8 was selected for further testing, grown-up in two further passages in IMDM containing 15% (vol/vol) FBS and cryopreserved in liquid nitrogen. Frozen aliquots once thawed were passaged in medium consisting of IMDM containing 10% (vol/vol) FBS lacking antibiotics.

Comparison of mitomycin C versus actinomycin D (AMD) as sensitizing agents

The lower limit of detection obtained with mitomycin C as the sensitizing agent was approximately 500 pg/mL recombinant human TNFα (data not shown). Substituting AMD for mitomycin C has been reported to shorten culture times and increase sensitivity to TNFs (Ostrove and Gifford, 1979; Kramer and Carver, 1986; Matthews and Neale, 1987). As predicted, substituting AMD for mitomycin C further increased the sensitivity of L929-8 cells to TNF (to approximately 60 pg/mL, an improvement of 8.3-fold in sensitivity) and decreased the assay time from 76 to approximately 24 hours (data not shown).

Comparison of MTT versus neutral red for assessment of culture viability

Although colorimetric analysis of viable cell content by MTT reduction is well established (Mosmann, 1983; Branch and Guilbert, 1986), we sought an agent which was noncarcinogenic and lacked the technical problems associated with MTT (Branch and Guilbert, 1986). Neutral red, already used in this capacity for TNF cytolytic assays (Rich *et al.*, 1989) appeared a promising candidate. Substituting neutral red for MTT did not alter the lower limit of detection of TNF but did steepen the dose-response curve, thereby increasing half-maximal (50% inhibition of L929-8 cell viability) sensitivity approximately 2-fold (Figure IV-1). Since this effect on half-maximal inhibition was reproducible in 2 additional experiments, we switched to neutral red for colorimetric analysis of viable content.

Effect of serum concentration on sensitivity to TNF

The biological fluids to be assessed for TNF content frequently contain appreciable levels of serum. Since it has been suggested that increasing serum levels in TNF bioassays decreases sensitivity (Kramer and Carver, 1986; Meager *et al*, 1989), the varying serum content in the sample could seriously affect the bioassay. We therefore determined the effect of varying levels of FBS on TNF sensitivity. Surprisingly, we found that L929-8 cells became more sensitive to TNF at higher FBS levels (Figure IV-2). In two independent experiments the concentration of TNF giving half-maximal inhibition was decreased approximately 4 to 5-fold by increasing the concentration of FBS from 5% (vol/vol) to 15% (vol/vol). Furthermore, the lower limit of detection of TNF was decreased by approximately 8-fold, from 15.8 pg/mL to 2 pg/mL. This may indicate that higher serum concentrations maintain L929-8 cells in a healthier state where these cells not only can provide a larger signal to noise ratio, but more sensitively respond to the inhibitory activity of TNFα. Indeed, it has been suggested that active protein synthesis is required for the observed TNF effects (Ostrove and Gifford, 1979 Chen *et al.*, 1987; Baloch *et al.*, 1990). Thus, in contrast to a previous report using a different subclone of L929 (L-M, Kramer and Carver, 1986), our results indicate that 15% (vol/vol) FBS containing medium optimally supports TNF induced cytolysis of L929-8 cells.

Effect of incubation temperature

It has been suggested that increasing the incubation temperature from 37°C to 39°C - 40°C may increase target cell sensitivity to TNF (Kramer and Carver, 1986; Yamazaki *et al.*, 1986; Niitsu *et al.*, 1988; Aderka *et al.*, 1989). Indeed, Figure IV-3 shows that increasing the temperature of the 16-hour incubation phase from 37°C to 40°C further increased the TNF sensitivity of L929-8 cells cultured in 15% FBS by approximately 4-fold. The concentration of human TNFα for half-maximal inhibition of L929-8 cell growth was decreased from 13.8 pg/mL to 3.6 pg/mL when the temperature was increased from 37°C to 40°C, and the lower limit of detection was decreased from 4 pg/mL to 0.5 pg/mL. Additional independent experiments (Table IV-2) confirmed this observation both with murine and human recombinant TNFα in that the lower limit of detection could be improved 1.7- and 3.9-fold, respectively, by increasing the temperature of incubation. Thus, 40°C was determined to be the optimal incubation temperature for the L929-8 bioassay.

Additional assay optimizations

Using AMD, 40°C incubation temperature and 15% FBS, we next asked what other parameters influence the sensitivity of the L929-8 bioassay. Figure IV-4 shows that manipulation of the cell number, concentration of AMD, or the length of time the L929-8 cells remain in continuous culture prior to being used for bioassay, have only minor effects on the sensitivity to TNFα. Thus, we have selected 5 x 10^4 cells per microwell and 2 μg/mL AMD for routine assays. We also have determined that L929-8 cells can be maintained at least 4 months in continuous culture at 2 passages per week without any significant loss in their TNF sensitivity. Although the TNF sensitivity of L929-8 cells was not greatly altered by different 40°C assay incubation periods, the magnitude of the signal was decreased at times greater than 20 hours (presumably due to the unhealthy effects of prolonged incubation with the transcriptional inhibitor AMD). Therefore, an incubation time of 16 - 20 hours was selected as optimal.

Comparison of L929-8 to parent L929 cells

Originally we found that L929-8 cells were more sensitive than their parent L929 cell line in the unoptimized bioassay using mitomycin C (Table IV-1). Interestingly, with the optimized AMD assay, the L929 parent cells detected TNF slightly more sensitively than the L929-8 subclone (Figure IV-5). However, magnitude of the response (the maximal absorbance obtained with medium alone compared to maximal TNF killing) with L929-8 cells was >2-fold better than with L929. On balance, we felt that the continued use of L929-8 warranted because of better signal to noise ratio.

Comparison of L929-8 and WEHI-164 clone 13 bioassays

The WEHI-164 clone 13 bioassay for the detection of TNF has been claimed to be the most sensitive assay currently available (Espevik and Nissen-Meyer, 1986; Eskandari *et al.*, 1990), although this claim has been questioned (Aderka *et al.*, 1989; Meager *et al.*, 1989). Therefore, we compared the optimized L929-8 bioassay to the WEHI-164 clone 13 bioassay as originally described by Espevik and Nissen-Meyer, 1986 (Figure IV-6). Although the lower limit of TNF detection was similar using either assay (2 pg/mL for L929-8 versus 3.98 pg/mL for WEHI-164 clone 13), the slope of the dose-response curve and thus, the concentration of TNF to induce half-maximal inhibition, was strikingly different. The dose-response curve of the L929-8 bioassay is steep with half-maximal inhibition of growth obtained at 8.5 pg/mL recombinant human TNFα. In contrast, the dose-response curve of the WEHI-164 clone 13 bioassay is much flatter with a half-maximal inhibition reached at greater than 1000 pg/mL recombinant human TNFα. Thus, the L929-8 bioassay detects low TNF levels with a much larger signal to noise ratio than does the WEHI-164 clone 13 assay and therefore quantitates lower TNF levels more precisely. However, the narrow concentration range of response requires sample dilutions to be tested.

Comparison of human and mouse TNFα

Figure IV-7 indicates the relative sensitivity of the L929-8 target cells to human and murine recombinant TNFα. As expected (Fransen *et al.*, 1986b; Kramer *et al.*, 1988), murine TNFα is a more potent inhibitory factor than human TNFα with the lower limit of TNFα detection being 500 fg/mL (29 fmol/L) and 200 fg/mL (12 fmol/L), respectively, for human and murine recombinant TNFα. These results also indicate that the optimized L929-8 bioassay for the detection of human TNFα is at least 2-fold more sensitive than any other currently available method, (Meager, *et al.*, 1989; Kobari *et al.*, 1990) including ELISA or RIA methods and has reproducible sensitivity for TNFs within the range of clinical usefulness (Beutler, 1990).

We have confirmed that the optimized assay detect natural TNFs from several animal species: human (supernatants from cytokine-stimulated human bone marrow cultures), rat (supernatants from LPS-stimulated exudate macrophages and spleen cells), and murine (supernatants from cytokine-stimulated bone marrow-derived macrophages, macrophage cell lines, placental cells and from LPS-stimulated RAW 264.7 cells) (data not shown). Natural TNF activity can also be detected in cell lysates and on intact, fixed cells.

Comparison of TNFα and TNFβ

It has previously been shown (Kramer and Carver, 1986) that certain murine fibrosarcoma cells are more sensitive to human TNFβ than TNFα. We also found that murine L929-8 cells are more sensitive to human TNFβ than to TNFα (Figure IV-8). In this experiment, the half-maximally inhibitory concentration of human recombinant TNFβ is approximately 6.5-fold lower than with human recombinant TNFα. Furthermore, the lower limit of detection is 130 fg/mL (7.0 fmol/L) for TNFβ compared to 500 fg/mL (29 fmol/L) for TNFα. Thus, the optimized L929-8 bioassay is not only very sensitive to TNFα but approximately 6-fold (on a molar basis) more sensitive to TNFβ.

Specificity testing

A number of cytokines and chemical agents (Table IV-3) were tested for their effects on L929-8 growth either alone or in combination with human recombinant TNFα. None of the agents tested alone had any effect on the growth of L929-8 cells using optimal assay conditions, nor did any alter the response to 5 pg/mL human recombinant TNFα (data not shown). Notable here was the observation that IFNτ, IL-1α, TGFβ, PGE_2, LPS, dexamethasone and cyclosporin A had no effect, alone or in combination with TNFα, since previous reports have suggested that TNF bioassay quantitations to be altered if any of these agents are in the assay sample (Fransen *et al* 1986a; Meager *et al.*, 1989; Eskandari *et al.*, 1990).

Stability of TNF

After optimal assay conditions had been established, we noted some variability in the level of TNF detection from assay to assay. Table IV-4 shows cumulative results obtained for 20 experiments using human recombinant TNFα. The half-maximal detection level was 4.45 ± 2.50 pg/mL and the lower limit of TNF detection was 1.32 ± 0.73 pg/mL. Although this variability was tolerable, nonetheless we asked whether it was in any way related to the storage conditions of the recombinant TNF used to generate the standard dose-response curves. Therefore, substocks at dilutions from 100 pg/mL to 100 ng/mL of human recombinant TNFα were prepared from a concentrated stock (2 x 10^4 ng/mL) which had been stored at -70°C. Dose- response curves were generated using these substocks after being freshly made or stored at 4°C or -20°C for 5 weeks. We found (Figure IV-9) that TNF at all the concentrations tested is very stable if stored frozen in 15% (vol/vol) FBS. However, at 4°C, even when stored in FBS-containing medium, TNF samples as concentrated as 100 ng/mL, lost potency. Additional experiments indicated that murine recombinant TNF was similarly unstable at 4°C but stable at -70°C, as was a source of natural murine TNFα (supernatant from LPS-stimulated RAW 264.7 cells; data not shown).

It would seem (Figure IV-9) that the optimized L929-8 bioassay is more reliable than the TNF standards and probably can be used to establish and maintain a standard TNF control. Thus, some of the variability noted in Table IV-3 was probably due to improper handling or storage of recombinant TNF standards. Repeat experiments, taking greater care in the storage and handling of the recombinant TNF used as a standard, indicated that less variance between assays and lower actual detection levels can be achieved (about a 2-fold increase in the apparent sensitivity compared to earlier work shown in Table IV-4). In 4 independent experiments using murine recombinant TNFα, the half-maximal inhibitory level was 0.58 ± 0.11 pg/mL (0.03 pmol/L) while in 2 experiments using human recombinant TNFα results of 2.40 and 2.24 pg/mL [Av = 2.32 pg/mL (0.13 pmol/L)] were obtained (See Figure IV-7). We conclude that TNF to be used as a standard should be aliquoted at >100 pg/mL and kept frozen until use. Furthermore, we recommend that these standards once thawed not be refrozen or used again to generate a standard dose-response curve. Likewise, samples for bioassay should not be refrigerated. Samples of biological fluids are best assayed on the same day as collection or stored frozen until the bioassay can be performed. Cellular lysates very rapidly lose TNF activity unless frozen (data not shown).

DISCUSSION

It has become apparent that antibody based assays such as RIAs and ELISAs detect both active and inactive TNF, thus, may not accurately measure true TNF activity (Beutler, 1990). *In vitro* bioassays can provide a rapid and sensitive means for the detection and quantification of various cytokines. However, currently available cytolytic or cytostatic bioassays for the detection and quantitation of TNFs, particularly human TNFα, are inadequate. It has been stated (Beutler, 1990) that for meaningful evaluation of the clinical role of endogenously produced TNF in chronic disease states, the lower limits of detection for TNF assays currently in use must be reduced at least 10-fold, down to approximately 0.1 pmol/L.

The bioassay described in this report, using a newly isolated subclone of L929 cells (L929-8), appears to satisfy all the requirements for a useful bioassay of TNF activity, especially in clinical settings. We describe an assay system which can reproducibly detect murine or human TNFα down to <50 fmol/L and human TNFβ down to 7 fmol/L. Other cytokines, including IL-1α, IFNγ and TGFβ, do not interfere with the accurate determination of TNF levels. The L929-8 cells are stable after multiple passages in continuous culture for prolonged periods. The assay is rapid and utilizes the noncarcinogenic agent neutral red for determination of viable cells. Furthermore, the assay is optimized for use with samples containing high serum concentrations, and its high sensitivity and specificity make possible detection and quantitation of TNFs in the presence of diluted human serum, which may contain specific or non-specific assay inhibitors. Use of this assay should allow for better clinical evaluation of TNF levels in certain disease states and should facilitate *in vitro* and *in vivo* studies of TNF using cell culture or animal samples where low TNF levels are suspected.

Table IV-1

Comparison of L929 subclones for sensitivity to human TNFα

Subclone	% Inhibition*
L929 - 1	85.7
L929 - 2	73.2
L929 - 3	65.8
L929 - 4	59.7
L929 - 5	27.8
L929 - 6	61.1
L929 - 7	82.6
L929 - 8	93.1
L929 - 9	91.1

* Inhibition to 50 ng/mL human TNFα determined as described in Materials and Methods.

Table IV-2

Effect of the temperature of incubation on the lower limit[§] of TNF detection

TNFα	Temperature of Incubation 37°C	 40°C	Fold Increase in Sensitivity
Murine[•]	8.75 ± 4.75[#] (n = 6)	5.25 ± 3.15 (n = 8)	1.7
Human[+]	2.95 ± 1.34 (n = 3)	0.75 ± 0.35 (n = 3)	3.9

[•] Using 5% FBS (vol/vol) and MTT readout.

[+] Using 15% FBS and neutral red readout.

[#] Mean ± SD (pg/mL)

[§] See Materials and Methods.

Table IV-3

Agents tested for detection by the optimized L929-8 TNF bioassay

Agent +	Source	Concentration(s)* Tested
IL-1α	human, pure recombinant; P. Lomedico, NJ	1.25 U/mL to 1250 U/mL
IL-2	human, pure recombinant; V. Paetkau, Edmonton	1000 U/mL
IL-3	murine, house prepared recombinant; L. Guilbert, Edmonton	1:2 dilution crude COS supernatant
IL-4	murine, pure recombinant; Immunex Corp., Seattle, WA	50 ng/mL
IL-5	murine, pure, recombinant; D. Kunimoto, Edmonton	100 U/mL
IL-6	human, recombinant; Genetics Institute, Boston, MA	15 U/mL to 1.5 x 10^5U/mL CHO supernatant
GM-CSF	murine, pure recombinant; Immunex; Glaxo	62.5 ng/mL
G-CSF	human, pure recombinant; Genetics Institute	124 ng/mL
CSF-1	human, pure recombinant; Genetics Institute	62.5 ng/mL
	murine, pure natural; E.R. Stanley, New York	2500 U/mL
LIF	human, pure recombinant; N. Gough, Melbourne	500 U/mL
TGFβ	porcine, pure; R. & D. Systems, Minneapolis, MN	25 ng/mL
NGF	murine, pure; R. Murphy, Edmonton	50 ng/mL
Epo	human, pure recombinant; Genetics Institute	25 U/mL
IFNγ	human, pure recombinant; Bio/Can, Mississauga	10 U/mL to 1250 U/mL
	murine, pure recombinant; American Cancer Society	100 U/mL
LPS	E. *coli*, strain 026-B6; Sigma Chemical, St. Louis, MO	0.78 μg/mL to 10 μg/mL
PGE_2	Sigma Chemical, St. Louis, MO	5 x 10^{-12}M to 5 x 10^{-5}M
Dexamethasone	Sigma Chemical, St. Louis, MO	2.5 x 10^{-11}M to 2.5 x 10^{-5}M
Cyclosporin A	Sandoz, Basel, Switzerland	7.8 ng/mL to 1000 ng/mL

+ Appropriate bioassays were employed to insure activity

* Agents were tested both alone and in combination with recombinant human TNFα using a suboptimal concentration to give approximately half-maximal inhibition of L929-8 cell growth.

Table IV-4

Sensitivity of the optimized L929-8 bioassay for the detection of human TNFα

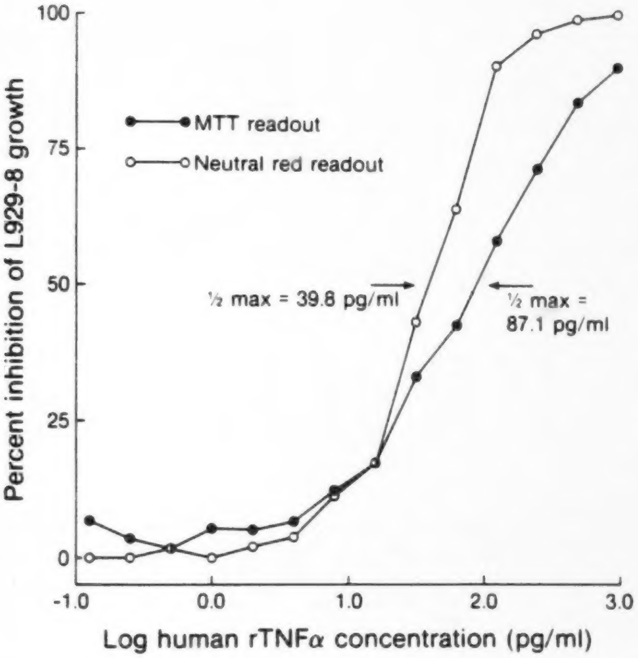
	TNF Concentration (pg/mL) for:	
Number of Assays	Half-maximal Inhibition of L929-8 Growth	Lower Limit of Detection[*]
20	4.45 ± 2.50[+] (range: 1.62 to 12.90)	1.32 ± 0.73 (range: 0.25 to 3.16)

[*] See Materials and Methods

[+] Mean ± SD

Figure IV-1

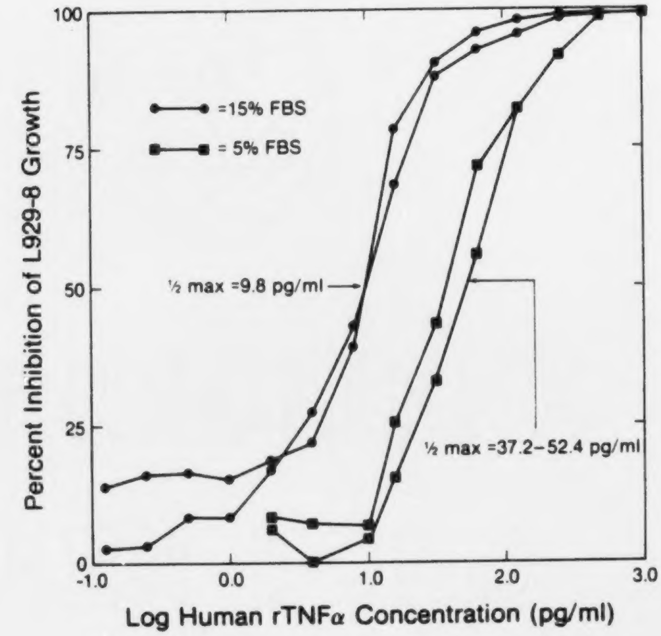
Comparison of MTT to neutral red



Comparison of MTT (●) to neutral red (○) for the determination of viable cell content. Results are presented as the percent inhibition of L929-8 growth (See Materials and Methods) versus the log human TNFα concentration. Each point represents the mean of quadruplicate determinations. Standard deviations were ≤15% of the mean.

Figure IV-2

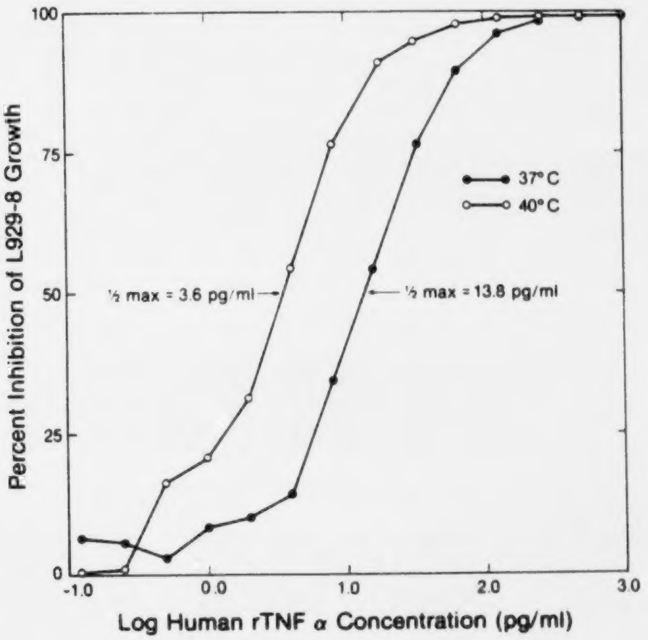
The effect of serum concentrations on the TNF sensitivity of L929-8 cells



The effect of serum concentration on the TNF sensitivity of L929-8 cells. Results are presented as the percent inhibition of L929-8 growth (See Materials and Methods) versus the log human TNFα concentration at fixed fetal bovine serum (FBS) levels of 5% (vol/vol; ■) or 15% (vol/vol; ●). Each point represents the mean of quadruplicate determinations. Standard deviations were ≤ 15% of the mean. Two independent experiments are shown.

Figure IV-3

The effect of temperature on the TNF sensitivity of L929-8 cells



The effect of temperature on the TNF sensitivity of L929-8 cells. Results are presented as the mean percent inhibition of L929-8 growth (See Materials and Methods) versus the log human TNFα concentration at 37°C (●) or 40°C (○). Each point represents the mean of quadruplicate determinations. Standard deviations were ≤ 15% of the mean.

Figure IV-4

Additional assay optimizations

Effects of varying: [A] the L929-8 cell number; [B] the concentration of actinomycin D (AMD); [C] the length of incubation; and [D] the time in continuous culture of the L929-8 target cell population prior to use in the bioassay on the TNF sensitivity of the optimized L929-8 bioassay [15% 9 (vol/vol) FBS, 40°C incubation temperature and using neutral red colorimetric assessment of viable cell content]. Individual symbols are as designated on the figure. Each point represents the mean of quadruplicate determinations. Standard deviations were generally $\leq$ 15% of the mean.

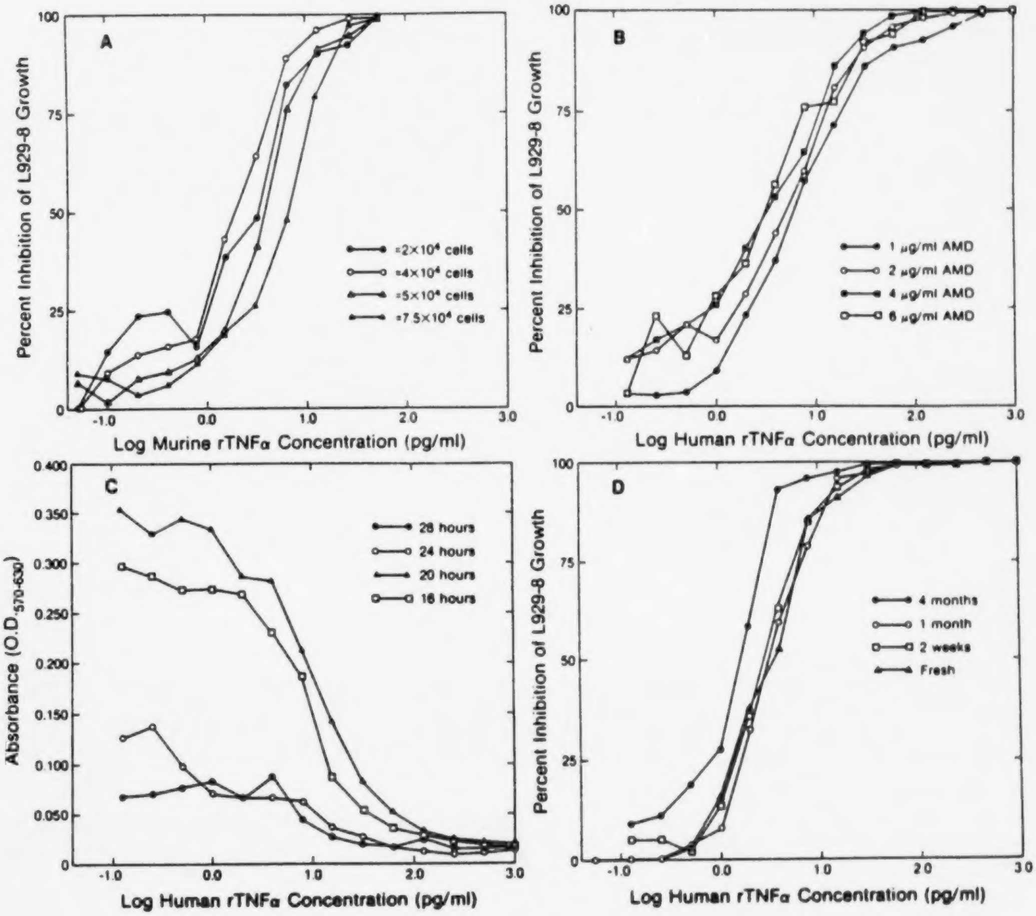
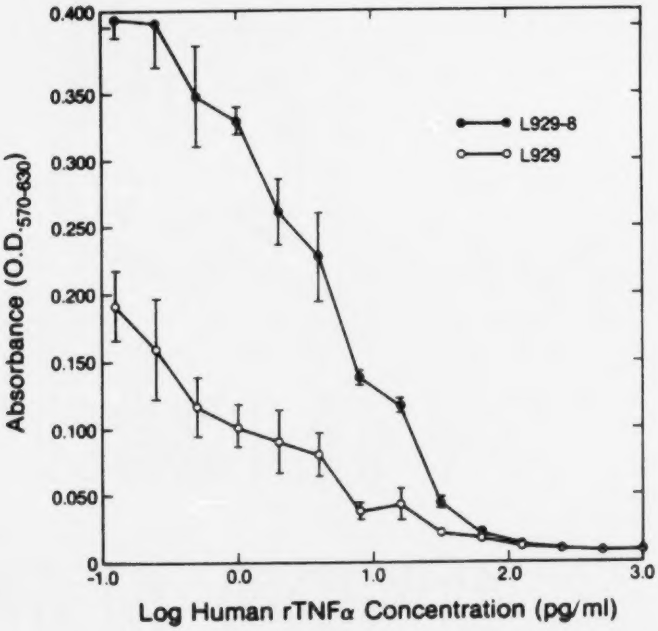

A
Percent Inhibition of L929-8 Growth
Log Murine rTNFα Concentration (pg/ml)
=2×10⁴ cells
=4×10⁴ cells
=5×10⁴ cells
=7.5×10⁴ cells
B
Percent Inhibition of L929-8 Growth
Log Human rTNFα Concentration (pg/ml)
1 μg/ml AMD
2 μg/ml AMD
4 μg/ml AMD
6 μg/ml AMD
C
Absorbance (O.D.570-630)
Log Human rTNFα Concentration (pg/ml)
28 hours
24 hours
20 hours
16 hours
D
Percent Inhibition of L929-8 Growth
Log Human rTNFα Concentration (pg/ml)
4 months
1 month
2 weeks
Fresh
0
25
50
75
100
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
-1.0
0.0
1.0
2.0
3.0

Figure IV-5

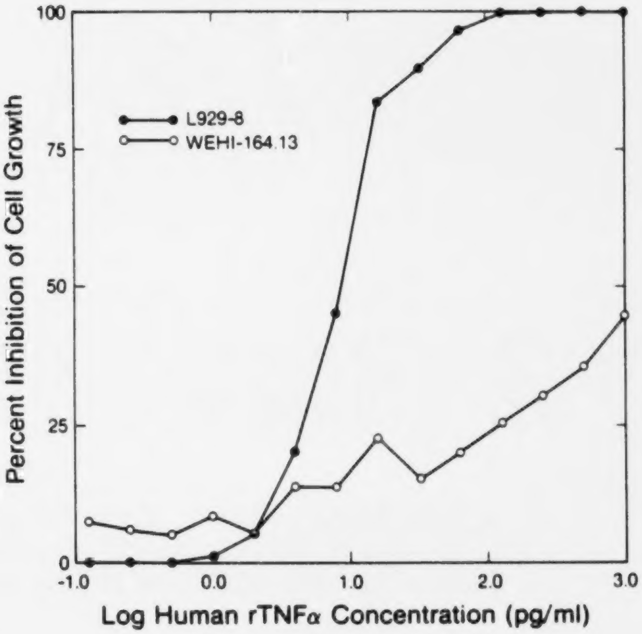
Comparison of L929-8 to parent L929 cells



Comparison of the TNF sensitivity of L929-8 subclone with the L929 parent cell line under optimized assay conditions [5×10^4 cells per microwell; 2 μg/mL AMD; 25% (vol/vol) FBS, 20 hours incubation at 40°C in 5% CO_2 in air; and using neutral red colorimetric assessment of viable cell content]. Results are plotted as the absorbance (optical density) versus the log human TNFα concentration for L929-8 (●) or L929 (○) cells. Data are represented as the means of quadruplicate determinations ± standard deviation.

Figure IV-6

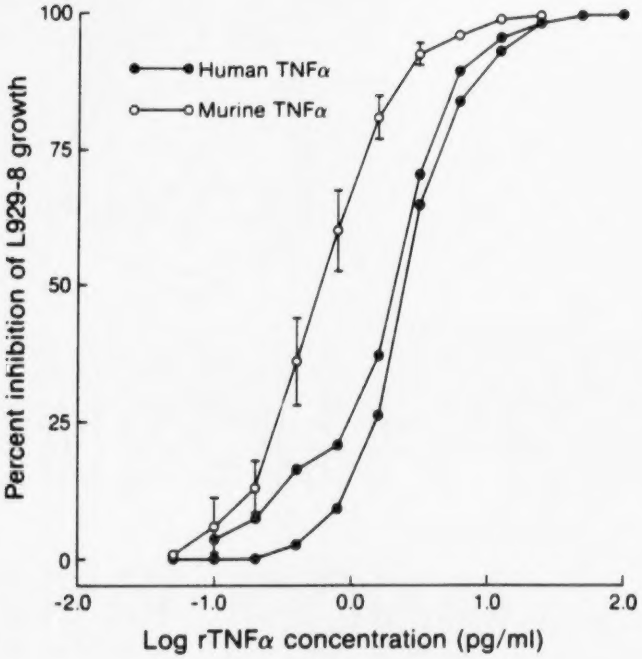
Comparison of the L929-8 with the WEHI-164 clone 13 bioassay



Comparison of the TNF sensitivity of the optimized (See Figure IV-5) L929-8 bioassay (●) with the WEHI-164 clone 13 bioassay (○). The results are plotted as the percentage inhibition of target cell growth versus the log human TNFα concentration. Each point represents the mean of quadruplicate determinations. Standard deviations were ≤ 15% of the mean.

Figure IV-7

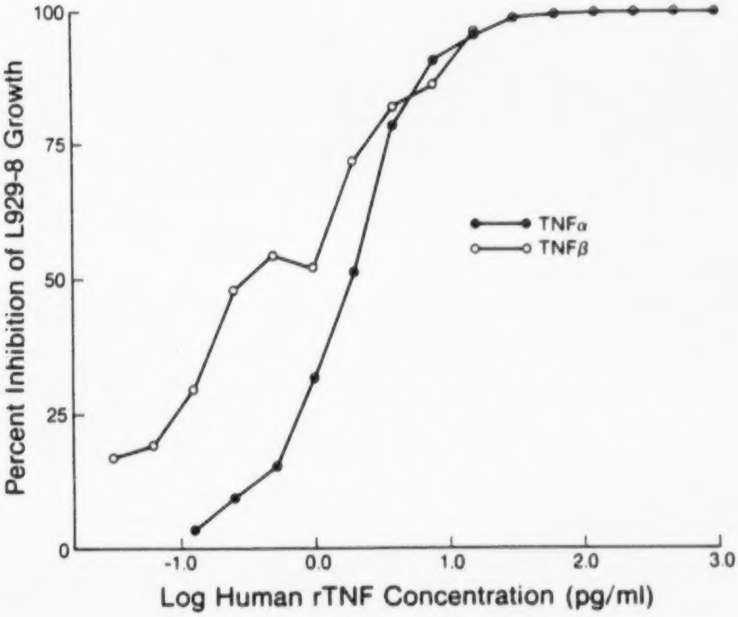
Detection of human and mouse TNFα



Comparison of the detection of human (●) and murine (○) recombinant TNFα by the optimized (See Figure IV-5) L929-8 bioassay. Particular attention was given to the storage conditions of the recombinant TNFs used to generate the dose-response curve (see last section Results). The murine results represent the cumulative mean ± standard deviation of 4 independent experiments. The human results show 2 independently generated dose-response curves which represent the mean of quadruplicate determinations (standard deviations were ≤ 10% of the mean).

Figure IV-8

Detection of human recombinant TNFα and TNFβ

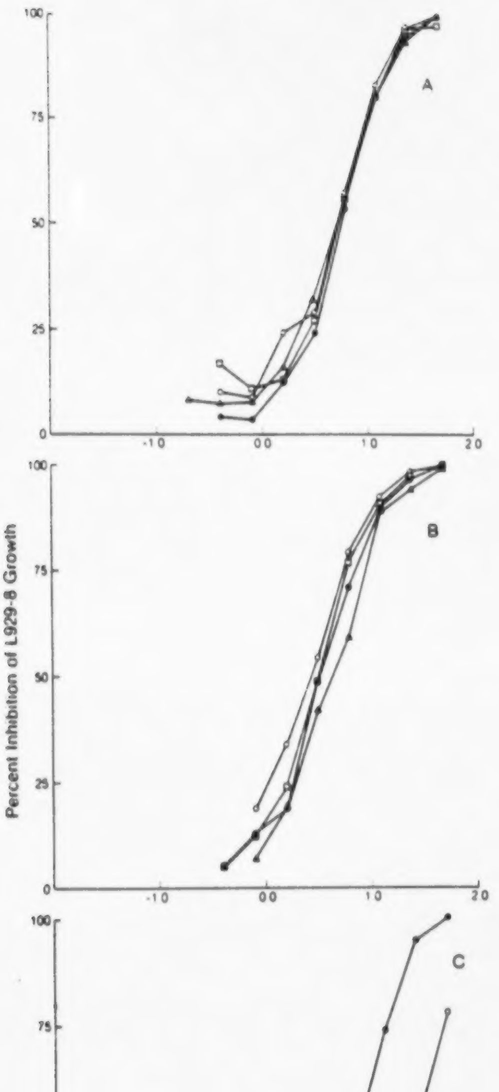


Comparison of the detection of human recombinant TNFα and TNFβ by the optimized (See Figure IV-5) L929-8 bioassay. Results are plotted as the percent inhibition of L929-8 growth versus the log TNF concentration, starting at 1000 pg/mL, for TNFα (●) or TNFβ (○). Each point represents the mean of quadruplicate determinations. Standard deviations were ≤ 15% of the mean.

Figure IV-9

The effect of storage conditions on the stability of recombinant human TNFα

The effect of storage conditions on the stability of recombinant human TNFα. Substock dilutions in IMDM containing 15% (vol/vol) FBS were made from a stock of TNF (1 mg/mL stored at -70°C) at: 100 ng/mL (●); 10 ng/mL (○); 1 ng/mL (△); and 0.1 ng/mL (□). Parallel substocks were stored as described below and used to generate dose-response curves starting at 50 pg/mL. Storage conditions were: [A]. Freshly prepared; [B]. -20°C for 5 weeks; or [C]. 4°C for 5 weeks. Results represent the means of quadruplicate determinations. The standard deviation was $\leq$ 15% of the mean.


A
B
C
100
75
50
25
0
-1.0
0.0
1.0
2.0
Percent Inhibition of L929-8 Growth
Log Human rTNFα Concentration (pg/ml)

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CHAPTER V

TUMOR NECROSIS FACTOR α PRODUCTION BY GROWTH-COMPETENT MOUSE MACROPHAGES AND POSSIBLE REQUIREMENT DURING MITOGENIC STIMULATION[1]

OVERVIEW

This chapter addresses some aspects of the specific thesis objective under Hypothesis II: to determine whether TNFα acts as an endogenous autocrine stimulatory molecule. I first examined TNFα expression at the mRNA level using Northern blot analysis which indicated that constitutive expression of TNFα mRNA was markedly increased after stimulation with CSF-1 or LPS (see Figure V-1). Further examination of biosynthetically labeled immunoprecipitated proteins showed multiple isoforms of TNFα, both associated with the cell (see Figures V-2, V-3 and V-4) or released into the culture medium (see Figure V-5). The molecular nature of these isoforms varied depending on the stimulus. Thus, LPS-stimulation produced three isoforms of 25 kD, 29 kD and 33 kD, CSF-1-stimulation produced two isoforms of 24 kD and 27 kD, while GM-CSF-stimulation resulted in the detection of a novel 20 kD isoform. Use of a highly sensitive bioassay (see Chapter IV), indicated that LPS-responsive C3H/HeN BMM were capable of higher TNFα production than LPS-hyporesponsive C3H/HeJ BMM or C3H/HeJ-derived S1 macrophages after stimulation with either LPS or CSF-1. After CSF-1 or GM-CSF stimulation of BMM, 68% to 94% of the TNFα was associated with the cell (see Table V-1). The CSF-1-

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[1] A version of this chapter has been submitted to the Journal of Immunology for publication. Branch, D.R. and Guilbert, L.J. Macrophage colony-stimulating factor (CSF-1)-stimulated production of tumor necrosis factor-α by growth-competent mouse macrophages.

stimulated proliferation of HeN BMM was significantly inhibited (an average of 28%; $p < 0.05$) with a neutralizing anti-TNFα antibody (see Table V-2). These results demonstrate that growth-competent, primary macrophages produce multiple isoforms of TNFα in response to external stimuli. Furthermore, the molecular nature and location (associated with the cell or released into the culture supernatant) of these isoforms may vary depending on the stimulus. Moreover, the data suggest that endogenously released TNFα may cooperate in an autocrine or paracrine/juxtacrine fashion with CSF-1 during mitogenic stimulation.

INTRODUCTION

TNFα is a cytokine that has a broad range of biological activities, target cells and sources (1-4). We have shown that one of the activities of TNFα is as a synergist that cooperates with the macrophage growth factor CSF-1 to stimulate the proliferation of primary mouse macrophages (5). This observation has been confirmed by other investigators (6, 7). Since CSF-1 has been reported able to induce TNFα production from mononuclear phagocytes (8), these observations suggest that TNFα may act as an autocrine agent that either enhances, or perhaps is essential to, CSF-1-stimulated macrophage proliferation. If so, TNFα would be the first cytokine to be documented to regulate normal hematopoiesis by an autocrine mechanism.

The effect of autocrine TNFα on proliferation stimulated by CSF-1 could be estimated with suitable genetic models that are deficient or defective in TNFα production. One such model has been documented by Beutler *et al* (9) who have shown that macrophages from C3H/HeJ mice accumulate TNFα mRNA in response to stimulation by high levels of bacterial cell wall LPS but release only minute levels of protein. However, it is not known whether macrophages of C3H/HeJ origin accumulate cell-associated TNFα after stimulation nor is it known whether the defect in these mice for TNFα release extends to stimuli other than LPS. There is also no published evidence that TNFα production is stimulated by CSF-1 in the absence of some form of prestimulation. The only report that CSF-1 stimulates TNFα production is that of Warren and Ralph (8) who demonstrated production from IFNγ primed human monocytes. Thus, whether CSF-1 can alone induce production of TNFα in growth competent mouse macrophages is not known.

These observations argue that little is known at the cellular or molecular level of normal cytokine-stimulated expression of TNFα protein and especially little is known of expression by mouse macrophages. The recent literature shows that TNFα is emerging as an important regulator of lymphocyte development (10) with possible roles in dysfunctions such as auto-immune diabetes (11-13). Many of these roles involve regulation of TNFα expression by cytokine networks in mechanisms not involving bacterial cell wall LPS-induced inflammation. Thus, the lack of detailed knowledge of TNFα expression impinges not only on the above-mentioned studies of autocrine regulation by this cytokine but also on studies of its paracrine, juxtacrine and endocrine roles in non-inflammatory situations.

The cDNA for mouse TNFα (14) predicts a 235 amino acid propeptide comprised of a 79 amino acid leader sequence and a 156 amino acid sequence that is ultimately released as the mature protein. However, the regulation of TNFα protein production may involve both post-transcriptional and post-translational modifications (14-18). Unlike human TNFα, there is one potential N-glycosylation site located within the mature mouse peptide (14). Different studies report mouse TNFα protein to be translated as a single propeptide having a molecular weight of 26 kD (19), 29 kD (20), or 50 kD (21, 22). However, more recent data indicate that mouse TNFα propeptide exists as multiple isoforms of 26 kD, 29 kD and 32 kD due to differential glycosylation (17). In addition, the propeptide leader sequence anchors the peptide in the cell membrane (14, 23). This membrane-associated TNFα protein can be cleaved, resulting in released forms which comprise a series of differentially glycosylated proteins having molecular weights of 17 kD, 18.5 kD and approximately 20 to 30 kD (15, 16, 24). Whether these isoforms may have varied function is unknown. However, it has been shown that the released 18.5 kD isoform is not glycosylated and does not induce lysis of TNFα-sensitive L929 fibroblasts (15). Most of the above documentation pertains to LPS-stimulated macrophages. The degree to which cell-associated TNFα is released in the absence of LPS stimulation is not known nor whether the same pattern of isoforms is expressed after cytokine stimulation in the absence of LPS stimulation.

We have therefore examined cytokine stimulated TNFα expression in macrophages of C3H/HeJ origin in order to minimize *in vitro* effects of LPS in culture venues. In order to control for possible genetic alterations in TNFα synthesis that may be related in a parallel fashion to their LPS defect, expression in HeJ macrophages is compared to expression in macrophages from C3H/HeN mice which differ from /HeJ animals at the single locus encoding the lps/lps co-dominant mutation (25). We show that stimulation with CSF-1 in the absence of IFNγ priming results in increased expression of both TNFα mRNA and biologically active proteins in either C3H/HeJ or C3H/HeN macrophages, that most of the protein expressed is cell-associated and that cytokines may stimulate expression of different TNFα isoforms. Finally, evidence is obtained that implicates an autocrine loop involving TNFα, which can be partially inhibited with neutralizing anti-TNFα antiserum, that suggests an important role for endogenous TNFα in the proliferation of mouse macrophages.

MATERIALS AND METHODS

Cells

Bone marrow-derived macrophages (BMM) from C3H/HeJ or C3H/HeN mice were prepared as previously described (5). Bone marrow cells were cultured 3 days with IL-3 and CSF-1 and then with CSF-1 for an additional 4 days. Nonadherent cells were selected on days 1, 3, and 5 of culture and adherent cells at day 7 of culture (d7 BMM) were harvested for proliferation and immunoprecipitation studies. The procedure yields a homogeneous population of growth factor-dependent cells of >98% macrophages (5). S1, a v-*myc*-transformed, growth factor-dependent C3H/HeJ macrophage cell line (5, 26), was used as early passage frozen aliquots which were thawed and recultured up to 1 month in Petri dishes containing Iscoves's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) supplemented with 15% (vol/vol) fetal bovine serum (FBS, Flow Laboratories, McLean, VA), 20% (vol/vol) L-cell conditioned medium and GM-CSF (5 ng/mL). The mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD.), was maintained in culture on plastic Petri dishes in medium consisting of RPMI-1640 (GIBCO) supplemented with 10% (vol/vol) FBS. L929-8 mouse fibroblasts were subcloned from L929 cells as previously described (27) and maintained in tissue culture flasks with IMDM supplemented with 10% FBS.

Cytokines

Pure, recombinant human CSF-1 (2.1×10^6 U/mL) was a gift of Genetics Institute, Boston, MA. Pure recombinant mouse GM-CSF (from Dr. R.J. Tushinski, Immunex Corp., Seattle, WA. and from GLAXO, Geneva, Switzerland), was diluted to 1 μg/mL in IMDM containing 15% (vol/vol) FBS and stored as previously described (26). Pure, recombinant mouse TNFα (1.2×10^7 U/mg) was obtained from Genentech, South San Francisco, CA. Lipopolysaccharide (LPS from *E. coli.*, strain 026-B6) was from Sigma Chemical, St. Louis, MO.

Neutralizing anti-TNFα

Neutralizing rabbit anti-mouse TNFα was from Genentech (lot 5896-95-15 K). The IgG fraction was purified from the whole antiserum by affinity chromatography with *Staphylococcus aureus* cell wall Protein-A (Protein-A Memsep, Millipore Ltd., Mississauga, Ontario). Briefly, 2 mL of whole rabbit anti-TNFα antiserum was dialyzed against buffer [1 M $(NH_4)_2SO_4$, pH 9.0) for 24 hours, then loaded onto a buffer equilibrated affinity column (0.2 mL/min) and recirculated 4 times. The column was washed with 10 bed volumes of buffer and the bound Ig eluted with 0.1M glycine, pH 2.5. Eluate fractions containing protein were pooled, dialyzed against 0.1M NH_4Cl, pH 7.0, lyophilized, and reconstituted in PBS. Preliminary studies showed that control rabbit IgG (Zymed Laboratories, So. San Francisco, CA) inhibited CSF-1-stimulated macrophage proliferation at concentrations above 200 ng/mL; thus, the protein A-purified rabbit anti-mouse TNFα and control rabbit IgG were added at a final concentration of 200 ng/mL for neutralization experiments. As measured by the TNFα bioassay (see below), 200 ng/mL of the protein A-purified IgG protein completely neutralized 50 pg/mL pure, recombinant mouse TNFα.

TNF bioassay

The L929-8 bioassay was used as previously described (27). Briefly, L929-8 cells were cultured in the wells of a 96-well flat-bottom microtiter plate at 5 x 10^4 cells per well in 50 μL of medium consisting of IMDM containing 15% (vol/vol) FBS plus 2 μg/mL Actinomycin D (Boehringer Mannheim, Laval, Quebec) for 2 hours at 37°C in 5% CO_2 in air. Fifty μL of supernatant, cell lysate, or recombinant mouse TNFα standards, were then added in triplicate or quadruplicate to appropriate wells and cultured at 40°C for 20 hours. Cell viability was assessed by incubation for 2 hours with 0.05% (wt/vol) in PBS of the dye neutral red, which is taken up by vital cells. Supernatant dye was removed, the adherent cells washed with PBS and color developed with 0.05M NaH_2PO_4 in 50% (vol/vol) ethanol. The optical density at 570 nm was measured on an automated microplate reader as previously described (26, 27). The lower limit of assay sensitivity is approximately 200 fg/mL using pure, recombinant mouse TNFα (27). This bioassay also detects TNFβ, but is not affected by other known cytokines (27).

Measurement of secreted and cell-associated TNFα

Samples for testing secreted and cell-associated TNFα were obtained as follows: 10^6 d7 BMM or S1 cells were plated into 35 mm tissue culture dishes in medium [IMDM containing 15% (vol/vol) FBS] and cultured overnight. The medium was discarded, the adherent monolayers washed four times with warm PBS, and 500 μL of fresh medium, containing 10 ng/mL CSF-1, 50 ng/mL GM-CSF or no growth factor, added to each dish. After 4 hours of culture at 37°C in 5% CO_2 in air, the supernatants were collected, centrifuged at 10,000 x g for 5-10 minutes, and tested the same day for TNFα. The remaining cells were washed 6 times with warm PBS, 300 μL of medium added to each dish, the adherent cells loosened with a rubber policeman and pooled in Eppendorf tubes, with a 300 μL plate rinse of fresh medium. The tubes were centrifuged (10,000 x g for 5 minutes), 300 μL of supernatant discarded, and the cell pellet resuspended in the remaining 300 μL of IMDM. The cell suspension was lysed by three cycles of freezing in liquid N_2 immediately followed by thawing with warm water. The lysates obtained in this fashion were assayed immediately for TNFα.

Immunoprecipitation

Previously described methods of immunoprecipitation (23, 28) were modified to allow for microanalysis. One million cells in medium lacking growth factors (IMDM containing 15% (vol/vol) FBS), were cultured overnight in 35 mm tissue culture dishes to form a quiescent confluent monolayer of cells. The cells were washed 4 times with warm PBS and 2 mL of serum-free, methionine and cysteine-free medium (RPMI-1640 select-amine kit, GIBCO) were added. After incubation for 2 hours at 37°C in 5% CO_2, the medium was discarded and 500 μL of fresh serum-free, methionine and cysteine-free medium added to each dish with appropriate cytokines. After incubation for 30 minutes at 37°C in 5% CO_2, 100 μCi ^{35}S-cysteine (Dupont Canada, Mississauga, ON; >600 Ci/mmol) and 100 μCi ^{35}S-methionine (Dupont Canada; >1000 Ci/mmol) was added to each dish. FBS (which had been extensively dialyzed against cysteine- and methionine-free medium) was added at a final concentration of 4% (vol/vol), and the cells incubated for 3 to 4 hours at 37°C in 5% CO_2. The supernatant was collected, made 1mM with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma), and saved for immunoprecipitation. After washing 4 times with PBS, the adherent cells were further processed at 4°C. The cells were lysed with 300 μL of ice-cold extraction buffer (0.02M Tris HCl, pH 8.0; 0.001M Na_2HPO_4; 0.137M NaCl; 0.5% NP-40) containing 1 mM PMSF and 0.01% globulin-free bovine serum albumin (BSA; Sigma). The lysate was collected into Eppendorf tubes, centrifuged for 1 minute at 10,000 x g and 40 μL of *Staphlococcal aureus*-protein A-Sepharose beads (SAS; Sigma), preblocked by suspension in PBS containing 1% (vol/vol) globulin-free BSA, was added. The mixture was gently rotated for 2 hours at 4°C, the samples centrifuged at 10,000 x g and the supernatant transferred to new Eppendorf tubes. Twenty μL of unpurified rabbit anti-mouse TNFα (final dilution 1 in 3750) was added and the samples slowly rotated at 4°C for 2 hours, at which time 40 μL of preblocked SAS was added and the sample slowly rotated for an additional hour. The sample was then centrifuged at 10,000 x g, the supernatant discarded, and the pellet washed 2 times with ice-cold wash buffer (0.1M Tris HCl, pH 8.0; 0.5M LiCl; 0.5% NP-40) containing 0.1% BSA and 3 times with ice-cold wash buffer without BSA. The tube was completely drained, 8 to 10 μL of sample buffer containing 2-mercaptoethanol added and the sample heated at 100°C for 5 minutes.

Supernatant samples prior to immunoprecipitation were dialyzed against PBS to remove any free radioactive methionine or cysteine and then lyophilized. After resuspension of the lyophilized material in 300 μL of extraction buffer, the samples were processed as above for the immunoprecipitation of TNFα from adherent cells.

After immunoprecipitation, samples were kept frozen and, prior to electrophoresis, centrifuged at 10,000 x g for 5 minutes. Electrophoresis was performed with PhastSystem (Pharmacia, Uppsala, Sweden), using 0.5 μL of sample on prepackaged homogeneous 12.5% or 20% SDS-polyacrylamide gels (Pharmacia). Pre-stained SDS-PAGE molecular weight standards (BIO-RAD, Richmond, CA) were used to estimate molecular mass. After electrophoresis, the gels were fixed for 15 minutes in 7% acetic acid followed by a 30 minute soak in an enhancement solution (AMPLIFY; Amersham Canada, Oakville, ON). After drying at 80°C for 30 minutes in a vacuum oven, gels were exposed to preflashed Kodak XAR 5 film with intensifying screens at -70°C for 10 to 30 days.

RNA extraction and Northern analysis

RAW 264.7 macrophages, S1 cells, or BMM were harvested, transferred to 100mm tissue culture dishes, and cultured overnight with the appropriate growth medium. S1 and BMM were washed, medium without CSF-1 added and the cells allowed to come to quiescence for 12 hours (S1) or 24 hours (BMM). Quiescent macrophages or RAW were then stimulated for 4 hours with medium alone (all cells), LPS (RAW and C3H/HeN BMM) or CSF-1 (S1 and C3H/HeJ

BMM). Total RNA was extracted according to the previously described protocol (29) using an acid guanidinium thiocyanate solution (4M guanidinium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1M 2-mercaptoethanol) and phenol-chloroform. RAW 264.7 macrophages, stimulated with 25 μg/mL LPS, was the positive control for TNFα mRNA while the TNF-sensitive L929-8 fibroblastoid cell line served as the negative control. For Northern blot hybridizations, 10 to 20 μg of total RNA was subjected to electrophoresis on a 1.2% agarose-formaldehyde gel for 18 hours at 8 volts, and a picture taken of the ethidium bromide stained ribosomal RNA bands. These bands were used to confirm that approximately equivalent amounts of RNA were loaded for each paired sample (cells stimulated with medium alone or with cytokine) and that there was no obvious degradation of RNA. RNA was transferred to a nylon transfer membrane (Biotrace RP, Ann Arbor, MI). Membranes were prehybridized at 42°C in 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.1% sodium dodecyl sulfate (SDS), 100 μg/mL denatured salmon sperm DNA, then hybridized for 18 hours in the same solution to which approximately 2.5 x 10^5 cpm/mL ^{32}P-labeled TNFα mouse specific cDNA (Genentech; ref. 14) was added. The probe was labeled to a specific activity of approximately 10^9 cpm/μg using hexanucleotide primers and [^{32}P]dCTP (Amersham Canada, Oakville, ON). Membranes were washed and, after drying, filters were exposed to Kodak XAR 5 film with intensifying screens at -70°C.

Statistical analysis

Statistics were done using the Analysis of Variance (ANOVA) and the Tukey multiple comparison analysis, usually referred to as the HSD (honestly significant difference) test (30, 31). A p value <0.05 was considered as significant.

RESULTS

Expression of TNFα mRNA

We first asked whether CSF-1 stimulated the expression of TNFα mRNA in macrophages of C3H/HeJ origin. RAW 264.7 cells, which secrete high levels of TNFα when treated with LPS (32) and LPS-responsive C3H/HeN BMM served as positive controls for TNFα mRNA expression in these studies. Northern analysis indicated that when cultured in medium alone, low, but significant mRNA for TNFα is present in all macrophage populations, with the possible exception of HeJ BMM (Figure V-1, top). As expected, stimulation with LPS resulted in an increase of mRNA for TNFα in both RAW 264.7 or d7 HeN BMM (Figure V-1, top). Likewise, stimulation with CSF-1 of d7 HeJ BMM or the C3H/HeJ-derived S1 cell line increased mRNA levels for TNFα (Figure V-1, top). The ethidium bromide staining for ribosomal RNA indicates that approximately equal amounts of total RNA was present for each paired analysis (Figure V-1, bottom). Thus, mRNA for TNFα is present in unstimulated C3H/HeN BMM, presumably as a result of the adherence of mouse macrophages to the tissue culture dishes (33) or due to low level induction by LPS or other contaminants contained in the medium. Nonetheless, this background level of TNFα mRNA can be increased after deliberate stimulation with either LPS (with HeN BMM or RAW 264.7) or CSF-1 (with HeJ BMM or S1 cells).

Expression of TNFα protein.

The above results demonstrate that BMM express TNFα mRNA after CSF-1 stimulation. However, it is known that TNFα mRNA levels do not necessarily correlate with protein production (9, 34) and that TNFα protein is expressed as multiple isoforms. Reports that a single species of rodent TNFα is produced also indicate widely varying molecular weights: 26 kD (19), 29 kD (20), and 50 kD (21, 22). More recent data argues that the propeptide form of TNFα exists as multiple isoforms of 26 kD, 29 kD, and 32 kD because of differential glycosylation (17). After cleavage of the 79 amino acid membrane anchoring region of the propeptide, a series of differentially glycosylated "released" proteins having molecular weights ranging from 17 kD to approximately 30 kD have been reported (15, 16, 18). However, the cell-associated propeptide is not always processed to the released, mature form (19, 23). Thus, analysis of cytokine stimulated TNFα production requires examination of both cell-associated (lysates) and released TNFα forms. Production was monitored by specific immunoprecipitation of biosynthetically labeled protein to determine the heterogeneity of recently synthesized TNFα and quantitated by a very sensitive bioassay.

Immunoprecipitation of biosynthetically labeled TNFα

Cell-associated TNFα

After stimulation with LPS, immunoprecipitation with specific anti-TNFα antiserum followed by SDS-PAGE revealed, in all macrophage populations, three protein bands having calculated molecular weights which varied slightly from assay to assay of 24-25 kD, 28-29 kD, and a broader range of 33-36 kD (see Figure V-2). These bands could be specifically competed for using an excess of nonradioactive recombinant mouse TNFα (data not shown; however, similar competitions are shown in Figures V-3 and V-4).

Further examination of immunoprecipitates by SDS-PAGE gels detected cell-associated TNFα in macrophage populations incubated in medium alone, CSF-1 or GM-CSF (Figures V-3 and V-4). When cells were stimulated by medium alone, CSF-1 or GM-CSF, two prominent species of TNFα were detected, one having a molecular weight of approximately 24 kD and one having a calculated molecular weight of 27 kD. As noted above, when cells were stimulated by LPS a third species having a molecular weight ranging between 33-36 kD was observed (Figure V-2). An isoform present at low levels, having a molecular weight of approximately 19-20 kD was observed only in GM-CSF-stimulated macrophages and was most evident with primary HeJ BMM (Figure V-3). These molecular species were all specifically competed using an excess of nonradioactive mouse TNFα (Figures V-3 and V-4).

Released TNFα

Cell supernatants also contained multiple forms of released TNFα.

Species of 20-27 kD, 17 kD and 14-15 kD were identified and were specifically competed with an excess of nonradioactive TNFα (Figure V-5). The band at 17 kD is the molecular weight species reported for the unglycosylated released form of mature TNFα (14, 15). The strong band at 14 kD may represent a proteolytic degradation product. However, the sharpness of the band argues for a specific low molecular weight species. The rather broad band detectable at 20-27 kD most likely represents the "ladder" of glycosylated TNFα isoforms previously reported (16, 17). Murine TNFα, unlike human, contains a site for N-glycosylation (14, 35) which may account for this diffuse pattern.

Bioactive TNFα

We quantitated the levels of biologically active TNFα protein associated with the cells or released into the culture supernatant using a highly sensitive bioassay (lower limit of detection for mouse TNFα of 200 fg/mL; ref. 27). Bioactive TNFα was detected in both supernatants and cell lysates from all macrophage populations. Table V-1 shows that under all stimulation conditions, S1 cells and HeJ BMM produce very low levels of TNFα protein (<20 pg/mL) of both secreted and cell-associated TNFα. HeN BMM constitutively produce much higher levels of TNFα than HeJ-derived macrophages. These results match our mRNA and immunoprecipitation results. With all macrophage populations, stimulation with high (5 μg/mL) levels of LPS resulted in more detectable TNFα than did either CSF-1 or GM-CSF treatment. The ability of the three macrophage populations to produce TNFα was in the order HeN BMM>HeJ BMM>S1 cells, regardless of the stimulus.

Cell-associated TNFα levels were higher than supernatant levels in all three macrophage populations. With either d7 HeJ BMM or the HeJ-derived S1 cell line, constitutive levels of both cell-associated and secreted TNFα were increased by CSF-1 stimulation and decreased by GM-CSF stimulation (Table V-1). In contrast, both CSF-1 and GM-CSF decreased constitutive levels of secreted TNFα from d7 HeN BMM. However, CSF-1 had little effect on cell-associated TNF levels, whereas GM-CSF dramatically decreased the levels of both cell-associated and secreted TNFα. Thus, as a general rule, significant levels of biologically active TNFα are found in CSF-1-stimulated cells. In contrast, GM-CSF appears to negatively regulate production of biologically active TNFα. LPS was by far the best stimulator of TNFα production inducing levels of TNFα which were 2- to 20-fold higher than those induced by CSF-1 and 6- to 350-fold higher than those induced by GM-CSF (Table V-1).

Effect of neutralizing anti-TNFα antibody on macrophage proliferation

In order to determine whether the levels of biologically active TNFα observed after stimulation with CSF-1 or GM-CSF significantly contribute to the mitogenic stimulation by these cytokines, we determined the effect of protein A-purified IgG preparations of neutralizing rabbit anti-mouse TNFα on cell proliferation. The antibody preparation significantly inhibited only CSF-1-stimulated proliferation of C3H/HeN BMM (Table V-2; $p<0.05$). The specificity of inhibition is in accord with the relative levels of released TNFα. That is, CSF-1-stimulated levels of released TNFα were higher than GM-CSF-stimulated levels; see Table V-1. Analysis of variance (ANOVA; ref. 30_) indicated that a significant difference ($p<0.01$) existed between 2 of 3 groups stimulated with CSF-1 but not with GM-CSF (Table V-2). However, ANOVA does not allow for a more specific conclusion, i.e., which means are different from which others. Therefore, the Tukey multiple comparison procedure was used. This method for pairwise comparisons appears to be more rigorous than other methods, particularly at the 0.05 level (36). Thus, endogenous TNFα appears to play, at least, a contributory role in the CSF-1-stimulated proliferation of normal, LPS-responsive mouse macrophages.

DISCUSSION

It has been shown that human monocytes can produce TNFα in response to the macrophage growth factors GM-CSF (37) and CSF-1 (8). The latter observation has been taken by many to indicate that CSF-1 induces TNFα production from mononuclear phagocytes. However, these investigators actually showed that pre-stimulation or "priming" with IFNγ was required prior to stimulation by CSF-1. Our results provide the first proof that CSF-1 can directly stimulate increases in both TNFα mRNA (Figure V-1) and TNFα protein (Table V-1) from macrophages. The lack of a pre-stimulation requirement in the present studies may relate to the use of different animal systems, human (8) versus mouse, or to the sensitivity of the TNFα detection system used in the present studies (27).

We show that LPS stimulates the production of three propeptide isoforms having molecular weights of 24-25 kD, 28-29 kD and a broader range of 33-36 kD. Since these isoforms are induced by LPS in either LPS-responsive C3H/HeN or hyporesponsive /HeJ mouse macrophages (Figure V-2), the results support the notion that the LPS defect of C3H/HeJ mice is incomplete (9, 38). Descoteaux and Matlashewski have shown that LPS stimulates expression of very similar isoforms from LPS-responsive BMM (39). We find that the high molecular weight isoforms were generally only seen after LPS stimulation (Figure V-2) and not seen, or were very faint, in unstimulated cells or after CSF-1 or GM-CSF stimulation (Figures V-3 and V-4). It has been shown that the three propeptide isoforms induced by LPS in RAW 264.7 macrophages are due to differential glycosylation (17) and it is thus likely that at least some of the heterogeneity of propeptide expression stimulated by LPS in the present study and in an earlier study (38), which used LPS-stimulated BMM from Balb/c mice, is also linked to glycosylation. In general, this study confirms these earlier findings and extends the observations to additional normal, primary macrophages stimulated with GM-CSF or CSF-1, including those of C3H/HeJ origin.

The results in this thesis suggest that LPS, CSF-1 and GM-CSF stimulate production of different cell-associated isoforms of TNFα in different macrophage populations. The finding that cytokines stimulated the production of a different lower molecular weight set of TNFα isoforms that does LPS in C3H/HeJ macrophages is intriguing. It is possible that LPS stimulates greater post-translational processing, possibly glycosylation, of TNFα propeptide than do CSF-1 or GM-CSF. Alternatively, during the 3-4 hours pulse used in these experiments, additional processing resulting in deglycosylation may have taken place in the CSF-1 or GM-CSF treated cultures (16). However, examination of the coding sequence for mouse TNFα suggests alternative explanations for the two very prominent, cell-associated isoforms of 24 and 27 kD expressed by unstimulated or CSF-1-stimulated C3H/HeJ and /HeN BMM (Figures V-3 and V-4). Firstly, different translation products may be possible. The Kozak 'scanning hypothesis' (40) predicts TNFα to have 3 possible AUG translation initiation codon sites: at nucleotides 144-146, 159-161 and 204-206 (14). This hypothesis would have initiation attempted at the 5' most proximal site (the site at nucleotides 144-146 suggested by Pennica *et al.*, 14), then in varying frequencies, to skip to more optimal downstream AUG sites. The site at 159-161 is not predicted to be more optimal than the first site and the resulting translation product would not be easily distinguishable from the 27 kD upstream start product. However, the AUG site at nucleotides 204-206 possesses the most optimal Kozak start site and thus would be predicted to be the initiation site for a sizable fraction of translations. The use of this site to initiate translation would result in the production of a second propeptide approximately 2.5 kD smaller than the full length propeptide thus generating the 24 and 27 kD isoforms. It has been suggested that multiple AUG initiation site codons are used in the translation of human TNFα (18). However, the proximal AUG site on the human sequence appears equally optimal as the next AUG site downstream; thus, translation isoforms may be less likely in this instance. The second alternative possibility is that there is serine protease cleavage at lysine -60/ methionine -59 in the propeptide sequence (corresponding to nucleotides 201-206) which would yield a product also 2.5 kD smaller than the full length propeptide. However, the presence of PMSF, a potent inhibitor of serine proteases, in the lysis buffer decreases the probability of such cleavage.

A unique molecular weight species of 19-20 kD was often detectable after stimulation with GM-CSF, usually with C3H/HeJ BMM, not C3H/HeN BMM and never with the S1 cell line (Figure V-3). This unique isoform could be due to GM-CSF regulated alternative splicing of the primary TNFα transcript. Indeed, Descoteaux and Matlashewski (39), showed that after stimulation of BMM with TNFα, but not LPS, two TNFα transcripts were detectable, however, no protein product of the second TNFα mRNA was found. Alternatively, the diffuseness of the 20 kD band in gel electrophoresis suggests the possibility of differential post-translational modification.

Taken together, the above findings suggest that the pattern of cell-associated TNFα isoforms depends on the nature of stimulation (by LPS, CSF-1 or GM-CSF) and on the target macrophage population (C3H/HeJ or /HeN). In contrast, we, like others (15-18, 24, 39), found multiple molecular weight forms released from the macrophage populations examined. Although no difference in the pattern of released isoforms was seen, the apparent molecular weights, as estimated from SDS-PAGE gels, differed slightly from those reported released from LPS-stimulated RAW 264.7 macrophages: others have reported 17 kD, 18.5 kD, and 20-27 kD isoforms (15-18, 24), while we could not demonstrate an 18.5 kD species and, instead, observed a prominent band at approximately 14 kD even in supernatants from LPS-stimulated RAW 264.7 cells (Figure V-5). It is likely that discrepancies in size between these and previous studies is due to differences in calibration of the SDS-PAGE gels.

We have previously shown that TNFα synergistically enhances CSF-1-stimulated macrophage proliferation (5) and we here show that mitogenically stimulated macrophages produce biologically active TNFα (Table V-1). The degree to which this endogenous production affects CSF-1- or GM-CSF-stimulated proliferation was determined by culturing in the presence of neutralizing antibody to TNFα (Table V-2). The observation that antibody inhibited proliferation in only one culture configuration (CSF-1-stimulated C3H/HeN BMM) is in accord with the relative production levels of biologically active TNFα (this configuration produced by far the highest levels, Table V-1) and with previous observations that TNFα does not cooperatively enhance GM-CSF-stimulated macrophage proliferation (5, 7). This result suggests that the other CSF-1-stimulated cultures either produce too little biologically active TNFα to affect proliferation or that the TNFα produced is inaccessible to neutralizing antibody. The inability of neutralizing antibody to inhibit CSF-1-stimulated proliferation of C3H/HeJ macrophages does not preclude autocrine interactions inaccessible to the antibody in these cell types. This is a distinct possibility given the cell-associated nature of TNFα expression in these cells after cytokine expression (discussed below). We have nonetheless shown that an autocrine loop that positively regulates proliferation can exist in normal hematopoietic cells. This is the first time such an observation has been documented.

Examination of the distribution of biologically active TNFα in cultures of growth-competent macrophages shows a large fraction to be associated with cells and the degree of cell-association appears to be primarily regulated by the nature of the stimulation. All over levels in cultures of C3H/HeJ cells was reproducibly low and predominantly cell-associated as shown in Table V-1. In contrast, levels in /HeN cultures varied widely, with basal levels sometimes high (as shown in Table V-1) and sometimes low (data not shown). GM-CSF always down regulated 'constitutive' levels in /HeN cultures whereas CSF-1 increased low basal expression and decreased high basal expression (the latter shown in Table V-1). In contrast to cytokines, LPS always upregulated 'constitutive' levels in /HeN cultures to more or less uniform upper levels (Table V-1 and data not shown). These results probably reflect varying amounts of LPS or LPS-like activities in the culture media. Thus, analysis of results from LPS-responsive /HeN cultures is difficult, but two trends are clear: (1) the cytokines regulate both levels and culture distribution of bioactive TNFα and (2) GM-CSF and CSF-1 regulate TNFα levels and distribution differently. Interestingly, LPS (and perhaps LPS-like conditions in the 'constitutive' situation) stimulates both production and release of TNFα such that the ratio of supernatant to cell-associated is less than one. The same trend is true of TNFα production in HeJ cultures: while cytokine treated cultures express more cell-associated than supernatant TNFα (sometimes considerably more), the LPS-stimulated cultures show the reverse. Although the regulation of culture compartment levels is complex and involves both input and egress, three conclusions can be abstracted from the data. (1) LPS strongly stimulates both production and release of TNFα, (2) GM-CSF and CSF-1 regulate culture levels and distribution differently, and (3) these cytokines, as contrasted to LPS, maintain expression of biologically active TNFα in a cell-associated manner. Taken together, the data suggests that *in vivo*, in the absence of strong bacterial or primary inflammatory signals, cytokines such as GM-CSF and CSF-1 maintain TNFα expression in macrophages in a discrete and private manner.

Table V-1

Production of TNFα by growth-competent mouse macrophages stimulated with macrophage growth factors

		TNFα Production⁺ ($pg/10^6$ cells) from:		
Stimulus	Form#	S1 Cells	HeJ BMM	HeN BMM
Medium	Secreted	<0.2	1.9	686
Medium	Cellular	1.5	9.8	581
CSF-1 (10 ng/mL)	Secreted	1.3	7.4	68
CSF-1	Cellular	1.9	21	595
GM-CSF (50 ng/mL)	Secreted	<0.2	0.96	4.1
GM-CSF	Cellular	0.72	2.0	14.8
LPS (5μg/mL)	Secreted	5.6	18.2	1500
LPS	Cellular	4.5	17.7	732

[+] Quantified using the L929-8 bioassay (27) by comparison to a pure, recombinant mouse TNFα standard; lower limit of sensitivity, 0.2 pg/mL.

[#] Secreted TNFα was measured after 4 hours of culture in supernatants conditioned by the indicated macrophage populations; cellular TNFα was measured in cell lysates (See Materials and Methods).

Table V-2
Effect of neutralizing rabbit anti-mouse TNFα on CSF-1- and GM-CSF-stimulated mouse macrophage proliferation

Cell Population	Stimulus	Stimulation Index (mean ± SD)	n	Significance (p value)
S1	CSF-1	0.91 ± 0.20	8	n.s.
HeJ BMM	CSF-1	0.99 ± 0.13	8	n.s.
HeN BMM	CSF-1	0.82 ± 0.14	8	<0.05
S1	GM-CSF	0.87 ± 0.24	5	n.s.
HeJ BMM	GM-CSF	0.96 ± 0.13	4	n.s.
HeN BMM	GM-CSF	1.00 ± 0.22	4	n.s.

10^3 cells/microwell were stimulated with 5 ng/mL CSF-1 or 62.5 ng/mL GM-CSF for 3 days in the presence of 200 ng/mL protein A-purified rabbit anti-mouse TNFα or 200 ng/mL purified rabbit IgG; 200 ng/mL of purified anti-TNFα can completely neutralize 25-50 pg/mL pure recombinant mouse TNFα. The stimulation index is the ratio of ^{3}H-TdR incorporation after culture of the rabbit anti-mouse TNFα to control rabbit IgG. Each separate experiment represents the mean ^{3}H-TdR uptake of 4 microwells; n indicates the number of independent experiments. The significance was determined using the Tukey multiple comparison analysis (31). ANOVA analysis of difference (30) gave greater significance ($p<0.01$).

Figure V-1

Northern blot analysis of TNFα expression in unstimulated, LPS- or CSF-1-stimulated mouse macrophages

Northern blot analysis of TNFα mRNA expression in unstimulated, LPS- or CSF-1-stimulated mouse macrophages. Quiescent BMM or S1 cells, or freshly passaged RAW 264.7 and L929-8 cells, were pulsed for 4 hr with the indicated stimulus and the total RNA extracted. Lanes 1-5 and 8-9 contain 20 μg total RNA. Lanes 6-7 contain 10 μg total RNA. L929-8 RNA (lane 1) serves as the negative control. RAW 264.7 RNA (lanes 2-3) serves as the positive control. Top: Total RNA was extracted from mouse macrophages, stimulated with medium alone (lanes 2, 4, 6, and 8), 25 μg/mL LPS (lane 3), 5 μg/mL LPS (lane 5) or 100 ng/mL CSF-1 (lanes 7 and 9), electrophoresed on a agarose-formaldehyde gel, and the RNA transferred to a nylon membrane, probed with a full length mouse TNFα ^{32}P-cDNA and the blot exposed to Kodak XAR 5 film for 5 days with intensifying screens as described in the Methods. Bottom: The ethidium bromide stained agarose-formaldehyde gel showing the 18S and 28S ribosomal RNA bands.

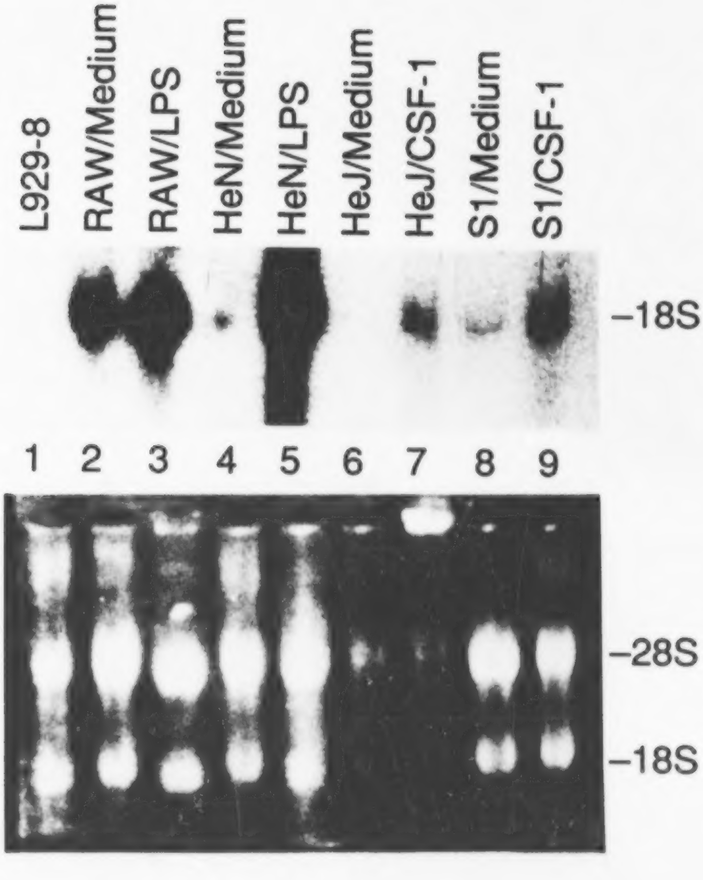
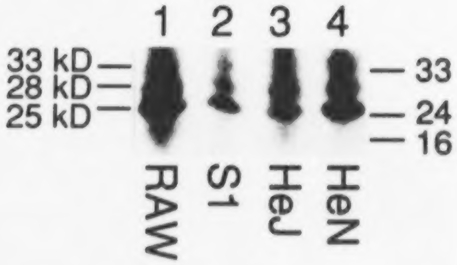

L929-8
RAW/Medium
RAW/LPS
HeN/Medium
HeN/LPS
HeJ/Medium
HeJ/CSF-1
S1/Medium
S1/CSF-1
–18S
1
2
3
4
5
6
7
8
9
–28S
–18S

Figure V-2

Immunoprecipitation analysis of TNFα produced by different mouse macrophage populations stimulated with LPS



Immunoprecipitation analysis of TNFα produced by different mouse macrophage populations stimulated with LPS: RAW 264.7 macrophage cell line (lane 1), C3H/HeJ-derived S1 macrophage cell line (lane 2), C3H/HeJ primary bone marrow-derived macrophages (lane 3), and C3H/HeN primary bone marrow-derived macrophages (lane 4). Cells were incubated with 5 μg/mL LPS for 30 minutes and then labeled with ^{35}S-methionine for 4 hours. Cell lysates were immunoprecipitated with rabbit anti-mouse TNFα and analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels. Autofluorography using pre-flashed Kodak XAR 5 film with intensifying screens was carried out at -70°C for 30 days. The position of molecular weight markers are shown on the right, and TNFα immunoreactive bands are identified by their apparent molecular weights on the left. These bands were specifically competed for by using an excess of nonradioactive mouse TNFα (data not shown; however, similar competitions are shown in Figures V-3 and V-4).

Figure V-3

Immunoprecipitation analysis of TNFα produced by C3H/HeJ bone marrow-derived macrophages

Immunoprecipitation analysis of TNFα produced by C3H/HeJ bone marrow-derived macrophages (BMM). **A.** Cells were incubated with medium alone (lane 1), 100 ng/mL CSF-1 (lanes 2 and 3) or 20 ng/mL GM-CSF (lanes 4 and 5) for 30 minutes and then labeled with a pool of ^{35}S-methionine and -cysteine for 3 hours. Rabbit anti-mouse TNFα (lanes 1,2 and 4) or anti-mouse TNFα mixed with an excess of nonradioactive mouse TNFα (lanes 3 and 5) was used to immunoprecipitate cell lysates and the precipitates analyzed by electrophoresis using 12.5% SDS-polyacrylamide gels. Autofluorography was done using preflashed Kodak XAR 5 film with intensifying screens at -70°C for 10 days. The position of molecular weight markers are shown on the left, and TNFα immunoreactive bands are identified by their apparent molecular weights on the right. **B.** A separate experiment using immunoprecipitation analysis of TNFα produced by C3H/HeJ BMM illustrating the 20 kD protein obtained only after stimulation with GM-CSF. Cells were incubated with medium alone (lane 1), 5 μg/mL LPS (lanes 2 and 3), 100 ng/mL CSF-1 (lanes 4 and 5), or 20 ng/mL GM-CSF (lanes 6 and 7) for 30 minutes and then labeled with a pool of ^{35}S-methionine and - cysteine for 2.5 hours. Rabbit anti-mouse TNFα (lanes 1, 2, 4, 6) or anti-mouse TNFα mixed with an excess of nonradioactive mouse TNFα (lanes 3, 5, 7) was used to immunoprecipitate cell lysates and the precipitates analyzed by electrophoresis using 12.5% SDS-polyacrylamide gels. Autofluorography was done using preflashed Kodak XAR 5 film with intensifying screens at -70°C for 9 days. The position of molecular weight markers are shown on the left, and the 20 kD TNFα immunoreactive band, induced by GM-CSF is indicated on the right (lane 6). This band is specifically competed for using an excess of nonradioactive mouse TNFα (lane 7).

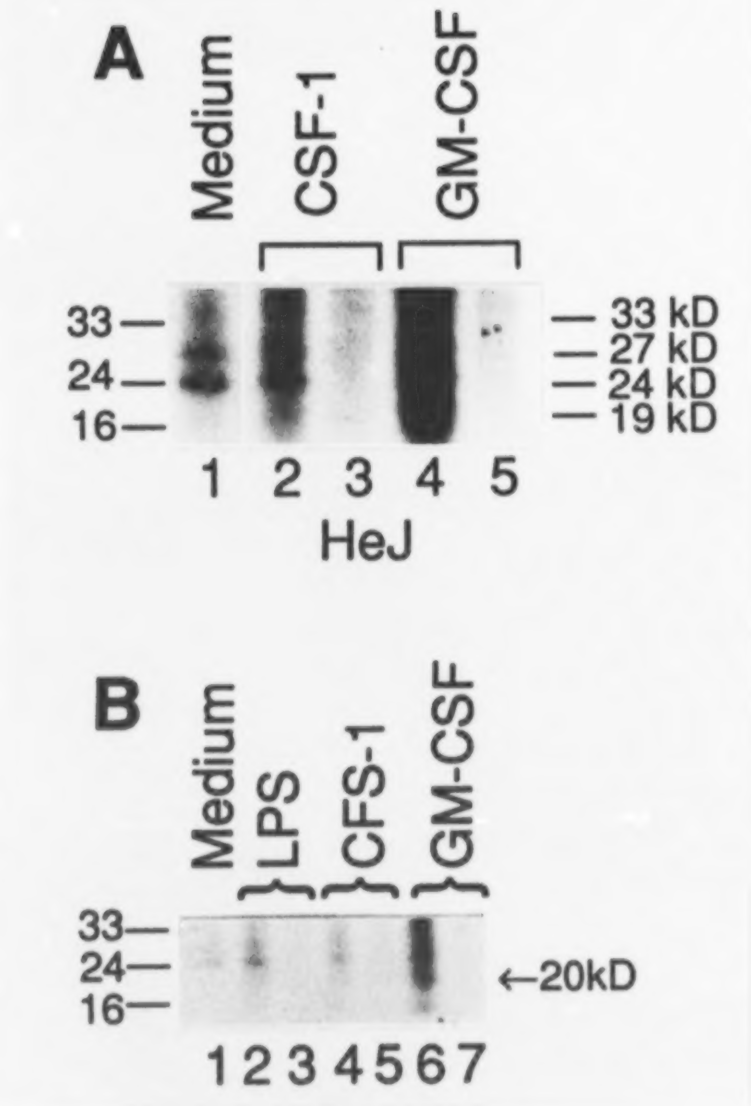
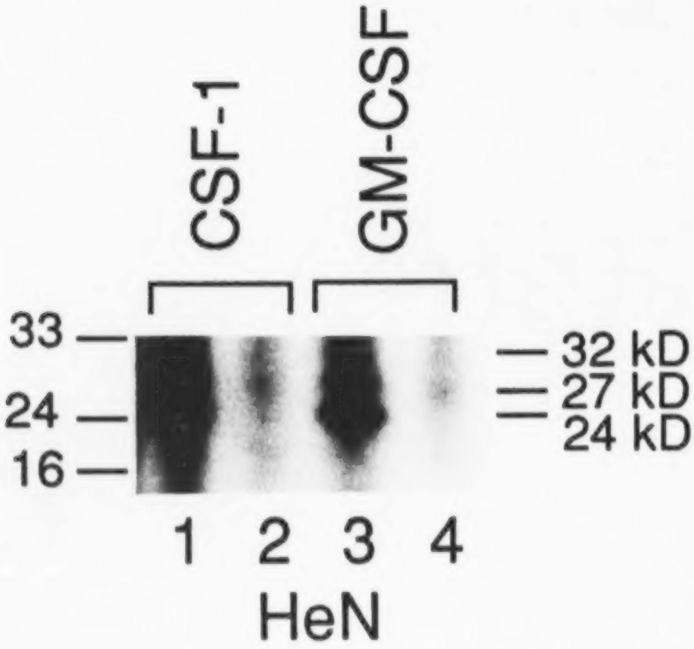

A
Medium
CSF-1
GM-CSF
33
24
16
33 kD
27 kD
24 kD
19 kD
1 2 3 4 5
HeJ
B
Medium
LPS
CFS-1
GM-CSF
33
24
16
←20kD
1 2 3 4 5 6 7

Figure V-4

Immunoprecipitation analysis of TNFα produced by C3H/HeN bone marrow-derived macrophages

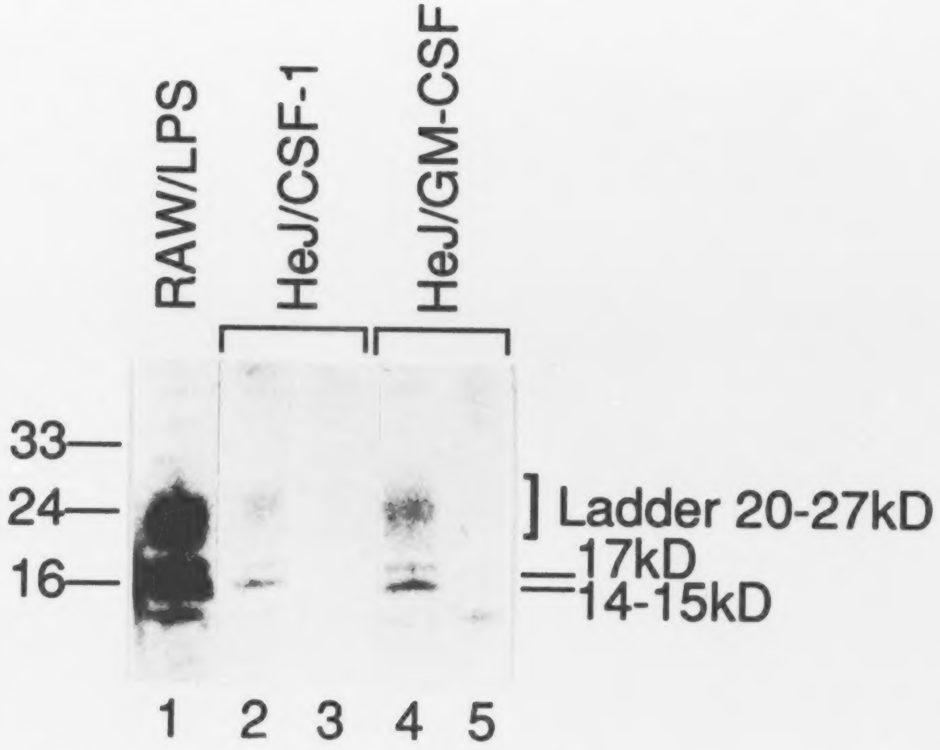


Immunoprecipitation analysis of TNFα produced by C3H/HeN bone marrow-derived macrophages (BMM). Cells were incubated with 100 ng/mL CSF-1 (lanes 1 and 2) or 20 ng/mL GM-CSF (lanes 3 and 4) for 30 minutes and then labeled with a pool of ^{35}S-methionine and -cysteine for 3 hours. Rabbit anti-mouse TNFα (lanes 1 and 3) or anti-mouse TNFα mixed with an excess of nonradioactive mouse TNFα (lanes 2 and 4) was used to immunoprecipitate cell lysates and the precipitates analyzed by electrophoresis using 12.5% SDS-polyacrylamide gels. Autofluorography was done using preflashed Kodak XAR 5 film with intensifying screens at -70°C for 10 days. The position of molecular weight markers are shown on the left, and TNFα immunoreactive bands are identified by their apparent molecular weights on the right.

Figure V-5

Immunoprecipitation analysis of released TNFα produced by RAW 264.7 macrophages and C3H/HeJ bone marrow-derived macrophages

Immunoprecipitation analysis of released TNFα produced by RAW 264.7 macrophages (lane 1) and C3H/HeJ bone marrow-derived macrophages (BMM; lanes 2-5). Cells were incubated with 5 μg/mL LPS (RAW 264.7, lane 1), 100 ng/mL CSF-1 (lanes 2 and 3), or 10 ng/mL GM-CSF (lanes 4 and 5) for 30 minutes and then labeled with a pool of ^{35}S-methionine and -cysteine for 4 hours. Rabbit anti-mouse TNFα (lanes 1,2 and 4) or anti-mouse TNFα mixed with an excess of nonradioactive mouse TNFα (lanes 3 and 5) was used to immunoprecipitate concentrated culture supernatant and the precipitates analyzed by electrophoresis on 20% SDS-polyacrylamide gels. Autofluorography was done using preflashed Kodak XAR 5 film with intensifying screens at -70°C for 20 days. The position of molecular weight markers are shown on the left, and TNFα immunoreactive bands are identified by their apparent molecular weights on the right.


RAW/LPS
HeJ/CSF-1
HeJ/GM-CSF
33
24
16
Ladder 20-27kD
17kD
14-15kD
1
2
3
4
5

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CHAPTER VI

GENERAL DISCUSSION

Each individual Chapter of the thesis contains specific comments within each Discussion section regarding this work. Within the overall general discussion I will only discuss the salient features of the thesis research and address the future directions which may be taken based on this work.

OVERVIEW

When the research reported in this thesis was initiated, it had been previously shown that CSF-1, GM-CSF and IL-3 were mitogenic for macrophages (Guilbert, 1985). Furthermore, the available data suggested that CSF-1 was the major mitogenic agent (Stanley *et al.*, 1983; Guilbert, 1985) and that it acted alone (Matsushime *et al.*, 1991). However, previous results with macrophage progenitors which showed that CSF-1 alone was not sufficient for proliferation (Guilbert, 1985) or that CSF-1 was not optimal (Williams *et al.*, 1986) suggested that multiple factors may be required for full mitogenic response. Thus, the role of CSF-1 in the proliferation of mouse macrophages was unclear. Furthermore, little was known about the interdependency of positive or negative regulatory cytokine pathways controlling macrophage proliferation.

The major objective of this thesis research was to assess the growth factor requirements for the proliferation of mouse macrophages. A central question was whether or not normal, mature, growth-competent mouse macrophages require multiple mitogenic signals for optimal proliferation and, if so, whether endogenously produced cytokines contribute to the observed proliferative response. During the initial phase of the studies, we examined, using well defined, highly homogeneous macrophages, various effects of exogenously added cytokines on macrophage proliferation (see Appendix 1a-1j), CSF-1 receptor binding (see Appendix 1k), and production of endogenous cytokines (see Appendix 1l-1s). These studies supported the contention that CSF-1 is the major mitogenic agent for mouse macrophage proliferation. However, it is important to note that the purification steps in the isolation of purified macrophages involves culture in the presence of CSF-1. Thus, CSF-1-responsive macrophages may have been selected or preferentially expanded during the purification process. Using macrophages derived by culture with other cytokines such as GM-CSF or IL-3 or mixtures of cytokines may result in the selection of a different population of macrophage-like cells (Young *et al.*, 1990; Metcalf and Nicola, 1992), possibly having different growth characteristics.

The salient features of this body of work are: (1) the isolation and definition of homogeneous mouse macrophage populations; (2) definition of the requirements for mouse macrophage proliferation which indicate that CSF-1 does not send an optimal mitogenic signal but may require a second signal provided by TNFα; (3) the apparent differential production and location of multiple TNFα isoforms from primary macrophages; (4) a proposed model for the regulation of mouse macrophage proliferation through a cytokine network which involves CSF-1, TNFα, and GM-CSF; and (5) the development of a highly sensitive bioassay for the detection of femtomolar levels of TNFα.

Isolation and definition of mouse macrophages

The significance of this work, at least in part, derives from the development of macrophage model systems for the study of stimulatory and inhibitory signals for mouse macrophage proliferation (see Chapters II and III). When this thesis work was initiated, a growth factor-dependent mouse macrophage cell line model was unavailable and primary cell systems, although available, had not been fully characterized. These studies have been aimed at better defining both primary and cell line macrophage populations to allow for a more precise examination of the potential regulatory activity of cytokines on mouse macrophage proliferation. Thus, the development, characterization, and use of highly defined, relatively homogeneous macrophage populations may enable a significant contribution to this area of investigation.

The major advantages of using the macrophage populations described in this thesis are as follows: (1) the primary cell populations are highly homogeneous being >92% macrophages by morphology and >82% by cell-surface antigen expression of the macrophage-specific marker F4/80; (2) the S1 cell line has similar morphological and cell surface antigen characteristics to the primary cells, including growth-factor dependence. Thus, identical results obtained with the primary cells and cell line rule out any contribution by non-macrophage accessory cells. The major disadvantages of these macrophage populations are: (1) Purified primary macrophages are not completely homogeneous due to variable, but relatively low, levels of contaminating non-macrophage cells (<20%) in the primary cell populations; (2) the macrophage cell line likely does not represent the normal physiologic state of resting macrophages; and (3) the presence of macrophage subsets in either the primary or cell line models. Nevertheless, use of both freshly isolated and cell line populations increases the ability to distinguish direct from indirect effects. Moreover, use of both cell populations may better determine whether a response seen using a cell line may, in fact, reflect the transformed nature of these cells or reflects the response of the normal counterparts.

Requirements for mouse macrophage proliferation

During this work, we have shown CSF-1 to have a distinct role in the proliferative response of mouse macrophages. Alone it provides the most potent signal for proliferation yet identified. However, CSF-1 does not provide an optimal mitogenic signal as addition of TNFα increases the response to maximally stimulatory levels of CSF-1 (see Chapter III; Branch *et al*., 1989). This observation has been confirmed by several other investigators (Shieh *et al*., 1989; Chen and Mueller, 1990).

The levels of exogenously added TNFα necessary for cooperation with CSF-1 in initial studies was high (125 ng/mL to 1250 ng/mL; see Table III-2) and likely nonphysiologic. Indeed, levels of TNFα production from our macrophage populations after stimulation by CSF-1 indicated that levels were orders of magnitude lower. Thus, whether the results obtained when using high levels of exogenous TNFα represent a potential for physiological relevance is unclear. However, in subsequent experiments, we could demonstrate that a similar cooperative effect of TNF with CSF-1 was seen at 10-fold less TNF concentration (see Appendix 1g). Furthermore, concentrations as low as 100 pg/mL TNFα were capable of synergistic activity with CSF-1 (data not shown). Thus, the concentration difference between exogenously added TNFα and the actual cellular production of TNFα detectable by bioassay may only differ by as little as 4-fold, when using BMM.

The amount of TNFα which is cell membrane-bound was not quantified in this work. However, after CSF-1 stimulation, the total cell-associated TNFα was found to be approximately 2 to 600 pg/mL depending on the macrophage population (see Table V-1). This cell-associated TNFα may be membrane-bound and biologically active (Kreigler *et al.*, 1988; Perez *et al.*, 1990). Therefore, depending on the rate of accumulation and release of membrane-bound TNFα, it is entirely possible that the relative concentration of TNFα on, or near, the cell surface may be much higher than the levels detectable in the culture supernatant or in total cell lysates. Furthermore, it is possible that the cell membrane-bound form of TNFα may arrange itself in such an array that may increase its biological activity, i.e., membrane-bound TNFα may more effectively crosslink and thus activate TNF-R1 and TNF-R2 on its own cell surface (autocrine effect) or on the cell surface of adjacent macrophages (juxtacrine effect). Thus, any apparent discrepancy in the levels of exogenously added TNFα necessary to elicit a proliferative effect, relative to the levels detectable in our cultures, may be resolved on the basis of an increased effective concentration and/or biologic activity of membrane-bound forms of TNFα.

Evidence for an autocrine/paracrine role for endogenously produced TNFα in macrophage proliferation was obtained using C3H/HeN BMM and neutralizing anti-TNFα antibodies (see Chapter V). However, the inhibitory effect of anti-TNFα on CSF-1-stimulated proliferation of C3H/HeN BMM was slight. This may be due to the inability of the neutralizing antibody to inhibit cell-associated TNFα, as most of the TNFα produced by these macrophages in response to CSF-1 (or GM-CSF) was found to be associated with the cell rather than released into the culture medium. Thus, endogenously produced TNFα may function as an intracrine growth factor. Indeed, previous investigators have provided evidence that intracellular signaling inducing a proliferative response can occur (Dunbar *et al.*, 1989). Using an IL-3 modified at its carboxy-terminal region so that, after introduction by retroviral gene transfer, the IL-3 would be retained in the endoplasmic reticulum, these investigators showed that this construct was able to induce the autocrine growth of an IL-3-dependent cell line, without detectable secretion of IL-3. Support for the hypothesis that TNFα may act by an intracrine mechanism comes from previous work which showed that the quantity of intracellular TNFα receptors can be three times higher than cell-surface receptors (Watanabe *et al*., 1988). Moreover, these intracellular TNFα receptors bind ^{125}I-TNFα with a Kd similar to that found for surface TNFα receptors (Watanabe *et al*., 1988). Thus, the inability of neutralizing anti-TNFα to markedly inhibit the proliferation of CSF-1-stimulated macrophages may be due to the high concentration of cell-associated TNFα acting through an intracellular receptor-mediated mechanism, inaccessible to the neutralizing effects of antibodies. An approach to examining this possibility would be to block the production of TNFα protein at a transcriptional or translational level and determine what effect this has on CSF-1-stimulated proliferation (see Future Directions, below).

It is difficult to determine with certainty whether the inhibition of C3H/HeN BMM proliferation when using anti-TNFα was due to inhibition of an autocrine or paracrine pathway mediated by endogenous TNFα. The neutralizing antibody may have inhibited released TNFα which may have been working as a paracrine factor acting on other adjacent cells. Alternatively, the antibody may have inhibited membrane-associated TNFα which was acting as a juxtacrine factor or may have inhibited released TNFα from binding to its receptor on the producing cell as an autocrine factor. The method used cannot distinguish among these possibilities. Even when using cell lines, it would be difficult to distinguish a paracrine/juxtacrine effect from an autocrine effect due to a potential for the presence of subsets within a given population of cells. For example, one subset may produce TNFα but not respond to it, while another subset may respond to TNFα but not produce it. Evaluation of single cells may be the only way to truly differentiate autocrine from paracrine effects.

In contrast to the results with C3H/HeN BMM, a lack of an inhibitory effect by neutralizing anti-TNFα antibodies when using C3H/HeJ cells indicated that regulation through paracrine/juxtacrine mechanisms involving TNFα do not play a significant role in the proliferation of these cells. This may be due to the very low levels of TNFα released from these cells or to the absence of cell-membrane material. Thus, the role of TNFα in proliferation may vary depending on the origin of the macrophages.

Production of TNFα isoforms from mouse macrophages

Differentially expressed isoforms of TNFα protein were identified during this thesis work. Multiple isoforms of TNFα propeptides and released TNFα have been reported previously using the macrophage cell line RAW 264.7 stimulated with LPS (Cseh and Beutler, 1989; Sherry *et al.*, 1990; Jue *et al.*, 1990). Similar isoforms have been shown to be produced by BMM in response to LPS (Descoteaux and Matlashewski, 1990), although the growth characteristics of these BMM were not specified. Thus, my results provide the first documentation for multiple TNFα isoforms produced by normal growth-competent macrophages using stimuli other than LPS. Furthermore, my data suggest that different patterns of isoforms may be induced with different stimuli. Thus, differential expression of cell-associated TNFα propeptide isoforms were observed following stimulation by LPS (3 isoforms), CSF-1 (2 isoforms) or GM-CSF (3 isoforms, including a novel 20 kD isoform). Moreover, the finding of apparently unique isoforms produced under specific conditions of stimulation suggest that these isoforms may have different functions.

The idea of distinct functions for different isoforms of TNFα is an intriguing possibility. Early on, investigators postulated that the amino acid leader sequence of TNFα may serve a specific function distinct from the actions of the mature peptide (Pennica *et al.*, 1985; Muller *et al.*, 1986). This speculation received support with the finding of a 26 kD propeptide membrane-associated isoform of human TNFα (Kriegler *et al.*, 1988). These investigators proposed that different forms of the TNFα molecule might induce different physiologic responses. They hypothesized that activated monocytes produce transmembrane TNFα (26 kD) at the site of inflammation and kill their targets by either a juxtacrine or local paracrine mechanism. In contrast, released TNFα (17 kD homotrimer) induces septic shock and cachexia. As these investigators also showed that the 26 kD molecule was the precursor of the 17 kD released TNFα, their hypothesis predicts that the release of TNFα is either differentially regulated or, as these authors suggested, regulated as local or systemic release of 17 kD TNFα. However, no experimental evidence was provided to support either possibility.

It may be that different isoforms of both cell-associated and released TNFα exist in all animal species to carry out the many distinct functions attributed to TNFα. Certainly, multiple isoforms of TNFα have been clearly shown to exist in murine (Cseh and Beutler, 1989; Jue *et al.*, 1990; Sherry *et al.*, 1990; Descoteaux and Matlashewski, 1990) and have been suggested in human (Muller *et al.*, 1986). Furthermore, an 18.5 kD isoform which was released from mouse macrophages was shown to be biologically inactive in the L929 cell cytotoxicity assay, while a 17 kD isoform was shown to be active (Cseh and Beutler, 1989). These investigators showed that the 18.5 kD species was a result of a 10 amino acid N-terminal extension of the 17 kD molecule and suggested that the retention of the 10 amino acids of the propeptide sequence was responsible for the inactive nature of this isoform.

In addition to the form of the TNFα, its location may also influence the function of these molecules. As stated above, it has been proposed that cell membrane-associated 26 kD TNFα may serve a distinct functional role from that of the 17 kD released TNFα (Kriegler *et al.*, 1988). In addition, the 17 kD isoform has been shown to function differently intracellularly as opposed to extracellularly. Microinjection of 17 kD TNFα was capable of killing cells which were normally resistant to contact with extracellular TNFα (Smith *et al.*, 1990). In another study, it was shown that endogenously produced TNFα served a protective function (Himeno *et al.*, 1990). These investigators used transfection of cDNA expression constructs containing TNFα in antisense or sense orientation. When the antisense product was expressed inside cells normally resistant to the cytolytic effects of exogenously added TNFα, these cells became sensitive. It was further shown that the sensitivity to the cytolytic effects of exogenous TNFα correlated with the absence of endogenous TNFα.

Any proposed differences in function may likely involve preferential interaction of the isoforms with the TNF-R1 or TNF-R2 receptors which likely send different intracellular signals (Tartaglia and Goeddel, 1992). This may be an explanation for the results showing that the released 18.5 kD isoform of mouse TNFα, although secreted as a soluble product, is biologically inactive in the L929 cell cytotoxicity assay (Cseh and Beutler, 1989). This isoform may not bind to the TNF-R1 receptor, proposed as being responsible for the cytolytic signal (Tartaglia and Goeddel, 1992), but may still bind to the TNF-R2 and send a different functional signal. No information regarding the binding of the 18.5 kD isoform to TNF receptors was given.

Taken together, the above argue that TNFα isoforms may have different functional activity depending on the molecular nature and location of the isoform. Thus, it is not unreasonable to believe that the numerous mouse TNFα isoforms detected in this and previous work may have differing physiological functions. The results of my thesis work which indicated that there may be differential expression and location of TNFα isoforms, which is dependent on the stimulus, may provide a model system to study whether these isoforms have different functional activities and/or show differing binding characteristics with TNF-R1 or TNF-R2 (see Future Directions, below).

Regulatory cytokine network

In addition to the evidence obtained in this thesis for the positive stimulatory effects of CSF-1 and TNFα on mouse macrophage proliferation, preliminary evidence was obtained for the negative regulation of CSF-1-mediated proliferation. In particular, GM-CSF appears to be a good candidate for a negative regulatory function (see Appendix 1b and 1c). Indeed, a recent report (Metcalf and Nicola, 1992), provides additional support for this supposition. Furthermore, GM-CSF has been reported to strongly down-regulate CSF-1R expression (see Appendix 1k; Walker *et al.*, 1985; Gliniak and Rohrschneider, 1990). Alternatively, GM-CSF may down-regulate endogenous production of TNFα (see Chapter V) which may be essential for the CSF-1 mitogenic signal. All of these considerations tend to support a dominant negative role for exogenous GM-CSF in regulating CSF-1-stimulated growth and/or proliferation of mouse macrophages.

In addition to GM-CSF, other cytokines showed inhibitory effects on the proliferation of our mouse macrophage models. These included G-CSF, IL-4, IL-6, TGFβ, IFNγ, and PGE_2 (see Appendix 1a, 1d, 1i, 1j). Other cytokines, particularly IL-10 (Fiorentino *et al.*, 1991; Ralph *et al.*, 1992), may also have inhibitory effects on our macrophage proliferation but were not examined.

Of the inhibitory cytokines and agents examined in this thesis, all have been shown in other studies (Walker *et al.*, 1985; Nedwin *et al.*, 1985; Aderka *et al.*, 1989; Hart *et al.*, 1989; Spengler *et al.*, 1989; Chantry *et al.*, 1989; Schindler *et al.*, 1990; Oppenheim *et al.*, 1990; Drapier and Wietzerbin, 1991; Appendix 1k) to down-modulate CSF-1R (GM-CSF, G-CSF, TGFβ, PGE_2), TNFα receptor or binding to receptor (IFNγ), and/or TNFα production (GM-CSF, IL-4, IL-6, PGE_2). Thus, the apparent down-regulatory effect of these agents on the proliferation of mouse macrophages is consistent with a mechanism of action affecting either CSF-1 or TNFα. This is compatible with the idea that the major positive regulators of mouse macrophage proliferation are CSF-1 and TNFα.

Taken together, the above information allows one to begin to formulate a model of a network of regulatory cytokines which mediate the control of mouse macrophage proliferation (see Figure VI-1). This regulation may involve both stimulatory and inhibitory molecules and potentially involves both paracrine and autocrine pathways. This working model proposes that CSF-1 and TNFα are the primary positive regulators of CSF-1-mediated proliferation while GM-CSF is the primary paracrine negative regulator. Thus, certain aspects of the normal control mechanisms for the proliferative response of mononuclear phagocytes is better understood as a result of this work.

Other findings

During the research for this thesis, it became necessary to develop a more sensitive bioassay for the detection and quantitation of TNFα protein (see Chapter IV). The development of this new bioassay has allowed for additional observations which are of significance.

Although it is generally accepted that CSF-1 can induce TNFα production from macrophages, this result was observed only after initial pretreatment of the macrophage population with IFNγ (Warren and Ralph, 1987). We have demonstrated that production of TNFα can be stimulated by CSF-1 in the absence of exogenous addition of IFNγ, thus providing the first evidence for CSF-1 directly stimulating TNFα production (see Chapter V).

Our results with macrophages derived from C3H/HeJ mice, which have been shown to be defective in their production of cytokines in response to LPS (see Chapter I), are supportive of one of the contentions of Beutler and coworkers (Beutler *et al.*, 1986), that C3H/HeJ macrophages have a defect in their ability to translate TNF-α mRNA. Under identical conditions of culture and stimulation, we found levels of TNFα protein were consistently lower when using C3H/HeJ compared with C3H/HeN macrophages, not only when stimulated with LPS, but also after stimulation with CSF-1. Furthermore, we confirm the observation of Beutler and coworkers (Beutler *et al.*, 1986) that macrophages derived from C3H/HeJ animals can produce minute amounts of TNFα when stimulated with LPS (the highest level we determined was <20 pg/mL). We extended this observation showing that the low levels of bioactive TNFα detectable from C3H/HeJ macrophages stimulated with CSF-1 are mainly associated with the cell rather than released into the culture media, while LPS appeared to stimulate the release of TNFα. Furthermore, the levels of cell-associated or released TNFα were consistently lower with C3H/HeJ-derived macrophages than with C3H/HeN macrophages. Thus, C3H/HeJ animals are not "unresponsive" to LPS stimulation or CSF-1 stimulation but are, in fact, hyporesponsive. Thus, although a translational block may be, at least in part, responsible for the apparent inability of C3H/HeJ animals to produce TNFα protein, our results argue that this potential translational block is incomplete and support the contention of others (Lattime *et al.*, 1988) that C3H/HeJ animals are capable of low-level TNFα protein production.

FUTURE DIRECTIONS

Mechanism of TNFα synergy effect

The mechanism by which TNFα and CSF-1 cooperate to increase the proliferation of mouse macrophages is not presently known. TNFα could cooperate with CSF-1 by (1) preventing apoptosis through the cell-cycle, thus allowing more cells to survive each cell-cycle passage; (2) directly affecting rate of progression through the cell-cycle; or (3) altering the number of cells entering the cell cycle. Since the synergistic effect of TNFα appears to be specific for CSF-1, it may be unlikely that the effect of TNFα would be in modulating apoptosis. It seems more likely that TNFα affects some aspect of cell-cycle entry or progression. Indeed, TNFα has previously been shown to perturb the cell-cycle status of L929 fibroblasts (Darzynkiewicz *et al.*, 1984), the HL-60 promyelomonocytic cell line (Darzynkiewicz *et al.*, 1987), and early hematopoietic progenitor cells (Warren *et al.*, 1990). In all these studies, TNFα acted to inhibit cell-cycle progression. In contrast, the synergistic effect of TNFα on CSF-1 proliferation may involve effects of TNFα on cell-cycle-specific proteins. Indeed, CSF-1 has been shown to induce novel cell-cycle specific cyclins (Matsushime *et al.*, 1991). Thus, the effects of TNFα may be on the induction of these cell-cycle regulatory proteins. Other proteins which influence cell-cycle progression such as cdc2 kinases, p53, and retinoblastoma (Rb) protein also may be influenced by TNFα.

It would be of interest to investigate the effects of TNFα on CSF-1-induced cell-cycle progression using propidium iodide and flow cytometric analysis, and also to examine the effect of CSF-1 and TNFα alone or in combination on the expression of cell-cycle-specific proteins. The latter experiments will require some means for inhibiting endogenous production of both TNFα and CSF-1.

Functional effect of inhibiting endogenous TNFα

Regardless of whether endogenously produced TNFα can cooperate with CSF-1 via a paracrine/juxtacrine or autocrine mechanism, the question remains whether endogenous TNFα is essential or important for the observed proliferative response to CSF-1 of mouse macrophages.

Synthetic oligonucleotides

A method using inhibition of the TNFα mRNA by antisense phosphorothioate oligodeoxynucleotides (S-oligos) was tried during this thesis research. 22-mer S-oligos made antisense to mouse TNFα mRNA starting at the 5'-proximal AUG initiation start site codon (5'-GGATCATGCTTTCTGTG-CTCAT-3') were found to inhibit in a sequence-specific manner (compared to a scrambled, base-matched nonsense S-oligo control sequence) TNFα protein production (see Appendix 2). The ability to effectively inhibit translation of TNFα mRNA was dependent on the rate of TNFα production by the target macrophages, which, in turn, was dependent on the activation history of the cells and paralleled their LPS-responsiveness to TNFα production.

Although these S-oligo sequences could markedly inhibit TNFα protein production in a sequence-specific manner, they could not be used to determine the effect of this inhibition on the CSF-1-stimulated proliferation of mouse macrophages. S-oligo sequences were found to inhibit, over a wide concentration range (2.5 μM to 40 μM), in an apparent nonspecific manner, proliferation of mouse macrophages (see Appendix 3). The mechanism of this inhibitory effect appeared complex but apparently was not related to toxicity, thymidine competition, or modulation of overall protein synthesis. It may have been related to the length and/or sulfurization of the S-oligos. Thus, further investigation of the mechanism of these nonspecific effects and their resolution is warranted before S-oligos can be used to address questions regarding the function of endogenously produced TNFα on the proliferation of mouse macrophages.

One could first examine whether the apparent nonspecific inhibitory effects seen with S-oligos is specific to this type of oligonucleotide sequence. The effect of using phosphorothioate, methlyphosphonate or unmodified antisense and nonsense sequences on inhibition of TNFα protein and proliferation of mouse macrophages could be tested. If the general inhibitory effect on macrophage proliferation is seen only when using S-oligos then other synthetic oligonucleotides may be useful in addressing the question of the role of endogenous TNFα. Alternatively, to preserve the increased resistance to degradation which S-oligos possess, different length sequences or modified S-oligo sequences could be tried. One advantage to using a synthetic oligonucleotide antisense approach is that the technique can be used in both primary cells and cell lines.

cDNA constructs

Expression vector constructs containing a full- or partial-length TNFα cDNA in sense and antisense orientation can be made (or obtained from other investigators such as Himeno *et al.*, 1990) and used to transfect the macrophage populations. Ideally, these constructs would have an inducible promoter, such as the heat-shock promoter. Thus, after permanent transfectants are established, expression of the sense and antisense cDNA can be induced and the effect on CSF-1-stimulated proliferation assessed. Although labor intensive, this approach to the inhibition of endogenously produced proteins is more likely to be successful, particularly when trying to block proteins which have low constitutive production and/or are inducible to relative low abundance.

Complementary to the above approach, one could transfect an antisense to the TNFα receptor(s) or a receptor antagonist construct, i.e., the TNFα receptor(s) truncated to lack the intracellular signaling domain. An additional approach may be to use a truncated TNFα receptor(s) which lacks the intracellular signaling domain and which has been further modified by introducing an oligonucleotide encoding a four-amino acid endoplasmic reticulum retention signal (Dunbar *et al.*, 1989), to trap TNFα within the cytosol.

One major disadvantage to using cDNA constructs is that these constructs can only be used with cell lines. Transient expression of these constructs in primary cells will likely not work for experiments to determine the function of endogenously produced TNFα since transiently expressed plasmids would occur, under the best of conditions, in < 10% of cells. This would preclude the use of primary cells since the nontransfected cells would likely continue to produce significant levels of TNFα. Moreover, selection based on antibiotic-resistance may be possible, however, during the time necessary to select the transfectants, these cells would likely differentiate to a nongrowing state.

In addition to determining the effects of inhibition of endogenously produced TNFα on cell proliferation, macrophage effector functions could also be studied. Evaluation of the effects of neutralizing anti-TNFα antibodies, antisense synthetic oligonucleotides or cDNA constructs on macrophage tumorcidal activity, Fc receptor-mediated phagocytosis, production of oxygen radicals, or resistance to infection by intracellular pathogens may show significant differences after inhibition of TNFα production and would complement the proliferation studies.

TNFα isoforms

It is presently unknown whether the different TNFα isoforms detected in the primary macrophage populations are due to differential glycosylation patterns, different or alternatively spliced mRNA, or to the use of multiple initiation start site codons. Furthermore, whether the different TNFα isoforms represent different functional proteins remains to be determined.

Using tunicamycin and glycanases it can be determined whether the multiple isoforms being detected are due to differences in glycosylation patterns, as has been shown for RAW 264.7 macrophages (Sherry *et al.*, 1990). RNAse protection can be used to determine by Northern blot analysis whether different mRNA transcripts may be present. RNAse protection will allow the detection of 1 base differences in mRNA transcript sequence. The hypothesis presented in Chapter V which suggests the different propeptide isoforms are due to multiple AUG initiation start site codons could be investigated as follows: (1) make expression vector constructs using a full-length TNFα cDNA and a truncated cDNA where the 5'-proximal AUG site has been deleted but the cDNA still contains the potential secondary AUG initiation start sites at positions -74 or -59. These expression vectors can be transfected into cell lines which do not normally produce TNFα, such as L929 fibroblasts, and the molecular weights for the expressed TNFα proteins determined by specific immunoprecipitation; (2) one could "tag" the cDNAs with a short oligonucleotide sequence which would result in a translation product which would consist of a fusion protein of TNFα/tag. Using an antibody to the tag may allow the TNFα isoforms to be easily harvested from large numbers of cells resulting in enough TNFα isoforms for protein purification and N-terminal sequencing. This latter approach could be used in mouse macrophages and thus avoid using a non-macrophage cell system such as L929.

Future studies aimed at addressing the functional role of the various isoforms would be of potential value, particularly the novel 20 kD isoform detectable after stimulation of macrophages with GM-CSF. Fusion proteins could be generated using a glutathione S-transferase (GST), Flag, hexahistidyl or other system. These fusion proteins could be made to consist of the "tag" plus the extracellular TNFα binding domain of either the TNF-R1 or TNF-R2. These fusion proteins could be used to "fish out" the TNFα isoforms in either supernatant or cell lysates. The proteins isolated in this way could be further purified on SDS-PAGE prepcell gels, the protein bands isolated in quantity to perform N-terminal sequencing, functional and receptor binding studies.

Another approach, although very labor intensive, would be to produce monoclonal antibodies to each of the different isoforms, purified as above, and determine the effect of depletion of one isoform versus the others on effector functions of TNF. The production of unique monoclonal antibodies to each of the isoforms will depend on these isoforms having distinct antigenic differences in carbohydrate moieties, N-terminal sequence or secondary structure, and that these different antigenic structures are immunogenic.

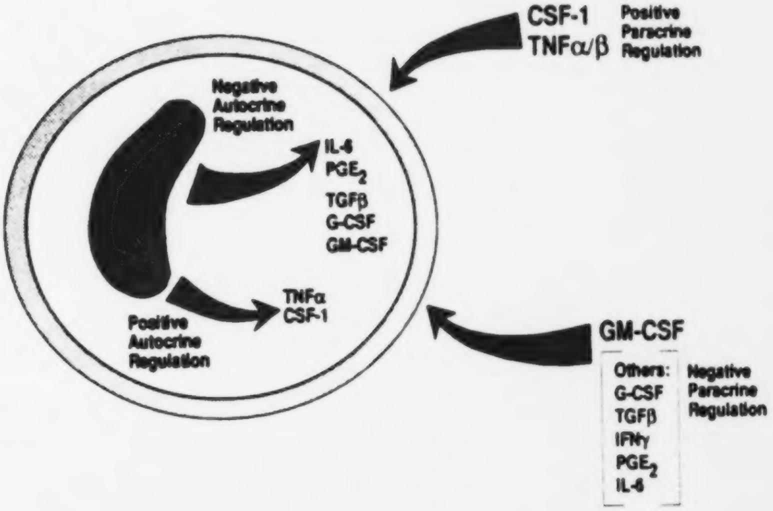
SUMMARY AND CONCLUSIONS

In summary, the data presented in this thesis provides the first evidence that relatively mature, growth-competent mouse macrophages require at least two signals for full mitogenic stimulation, supporting the original working thesis hypothesis. The finding that CSF-1 does not appear to send an optimal mitogenic signal and may require either exogenous (paracrine) or endogenous (autocrine/juxtacrine/intracrine) TNFα for an optimal proliferative response supports a general concept that cells require multiple signals for growth, as has been shown in other systems (see Chapter I). Although we did not obtain any evidence that demonstrated that CSF-1 cannot act alone, our results argue that CSF-1 alone does not send an optimal mitogenic signal to mouse macrophages in our culture system. Further work (see Future Directions, above) needs to be done in order to answer the question whether CSF-1 acts alone to stimulate mouse macrophage proliferation. Nonetheless, by both mixing experiments using CSF-1 and TNFα, and neutralizing anti-TNFα, we provide the first evidence for a potential regulatory mechanism, mediated by either exogenous or endogenous TNFα, for modulating the proliferation of normal growth-competent macrophages.

The conclusions from this thesis work are: (1) CSF-1 is the major mitogenic agent yet examined for mouse macrophage proliferation; (2) TNFα is necessary for the optimal proliferative response of mouse macrophages; (3) endogenously produced TNFα may contribute to the observed CSF-1-mediated proliferative response (HeN model); (4) GM-CSF appears to be a strong inhibitor of CSF-1-stimulated proliferation which may, in part, be mediated by a down-regulatory effect on endogenous TNFα production; (5) an essential role for endogenously produced TNFα cannot be ruled out as the absence of an intracrine mechanism was unable to be demonstrated (HeJ model); (6) LPS, CSF-1 and GM-CSF may regulate production of different TNFα propeptides; (7) in the process of doing this work, what is likely the most important, but unexpected, observation of the thesis was: that cytokines may regulate the release of TNFα, with CSF-1 and GM-CSF, in contrast with LPS, keeping most of the TNFα associated with the cell. This may be a homeostatic mechanism, since released TNFα can be dangerous to the host.

Figure VI-1

Cytokine regulation of CSF-1-mediated mouse macrophage proliferation


CSF-1
TNFα/β
Positive Paracrine Regulation
Negative Autocrine Regulation
IL-6
PGE2
TGFβ
G-CSF
GM-CSF
TNFα
CSF-1
Positive Autocrine Regulation
GM-CSF
Others:
G-CSF
TGFβ
IFNγ
PGE2
IL-6
Negative Paracrine Regulation

A proposed working model for the regulatory cytokine network controlling the proliferation of CSF-1-stimulated mouse macrophages. In this model, it is proposed that exogenous CSF-1 in combination with TNFα or TNFβ sends an optimal mitogenic signal, while exogenous GM-CSF is the major down-regulator of this mitogenic response. Furthermore, it is postulated that endogenously produced CSF-1 and/or TNFα may also serve to positively regulate the mitogenic response while the production of endogenous IL-6, TGFβ, G-CSF, GM-CSF or PGE_2 all may serve as autocrine down-regulators of the mitogenic response.

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APPENDIX 1

GROWTH MODULATORY EFFECTS OF CYTOKINES ON MOUSE MACROPHAGES

The following methods were used to examine the effects of endogenously added cytokines on macrophage proliferation, binding of CSF-1 to its receptor and production of endogenous growth-relevant cytokines.

Proliferation assays

Macrophages for proliferation assays were harvested from polystyrene Petri dishes following five minutes of treatment at 4°C with sterilized 5 mM ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS), and washed four times with sterile PBS (see Chapter III). One-thousand cells in 50 μL of IMDM containing 15% (vol/vol) FBS were added to 50 μL of the same medium containing cytokines in wells of 96-well flat-bottom microtiter plates and incubated at 37°C in an atmosphere of 5% CO_2 in air. Proliferation was quantitated by addition of ^{3}H-TdR (tritiated-thymidine, New England Nuclear, Boston MA, 20 Ci/mmol, 1 μCi/microwell) for the last 4 hours of a 3 day incubation period, and the cells harvested using a Titertek 550 cell harvester (Flow Laboratories) after the strongly adherent macrophages were loosened with 0.05% Triton X-100 in PBS for 5 to 10 minutes. ^{3}H-TdR incorporation was measured on a scintillation counter.

^{125}I-CSF-1 binding

As previously described (see Chapter III), CSF-1 was radio-iodinated with Chloramine T to approximately 3 x 10^{18} cpm/mol and the biologic activity of each iodinated preparation was determined by colormetric bioassay. Log-phase growing macrophages were harvested, washed four times with PBS, resuspended in IMDM containing 15% (vol/vol) FBS, and aliquoted at 2 x 10^4 per well per 50 μl in a 96-well flat-bottom microtiter plate. After overnight incubation at 37°C in the absence of growth factor to allow the cells to come to a resting, quiescence state cytokines or other agents were added to selected wells for 0.5 - 1.0 or 24 hours and specific receptor-mediated binding of ^{125}I-CSF-1 was measured as previously described (see Chapter III).

Macrophage supernatants

Unless otherwise indicated, supernatants were collected from BMM, or from S1 cells, harvested from Petri plates as described above, both cultured at 5 x 10^4 /mL for 3 days in 96-well plates in IMDM containing 15% (vol/vol) FBS, or cytokine, or combinations of cytokines. After culture, the microtiter plates were centrifuged at 1500 x g in a refrigerated centrifuge and the cell-free supernatants harvested for testing. Where indicated as Method 2, supernatants were harvested from BMM, grown and selected on 96-well tissue culture dishes as previously described (Tushinski *et al.*, 1982). Briefly, 2.3 x 10^4/mL primary d3 HeJ BMM or 1.3 x 10^4/mL primary d3 HeN BMM were cultured in 96-well microtiter plates for 2 days with CSF-1, the well contents decanted, the wells carefully washed twice with 150 μL warm medium (IMDM containing 2% [vol/vol] FBS), then 100 μL of growth medium containing CSF-1 at 3000 U/mL was added to each well. After incubation for 2 more days at 37°C in 5% CO_2, the supernatant was decanted, the cells washed twice with medium and 100 μL IMDM containing 15% (vol/vol) FBS added for a 20 hour incubation. Supernatants were again decanted and 100 μL of medium alone, or medium containing cytokines, or combinations of cytokines was added to selected wells. After 24 hours, these supernatants were collected and centrifuged 10 minutes at 10,000 x g, to remove debris, prior to testing for endogenously produced cytokine activity. In a similar fashion, S1 cells were cultured in 96-well microtiter plates at 5 x 10^5/mL in medium lacking growth factors for 2 hours, the supernatant was decanted, 100 μL of medium alone, or medium containing cytokines, or combinations of cytokines added, and after 24 hours of culture at 37°C, the supernatants were collected, centrifuged as above and the cell-free supernatant saved for testing.

DA-1k assay

IL3, GM-CSF, G-CSF, Epo, and LIF all stimulate proliferation of murine DA-1k cells (Branch and Guilbert, 1986; Branch *et al.*, 1987; Croy *et al.*, 1991). GM-CSF in macrophage supernatants was quantitated by the specific neutralization of supernatant stimulation of DA-1k cell proliferation by specific anti-GM-CSF neutralizing antibody. DA-1k cells were plated at 10^4 cells/well in 96-well microtiter plates with macrophage culture supernatants or cytokine controls that had been preincubated with rabbit anti-murine GM-CSF or NRS (end concentration, 1%) for 1-2 hours at 37°C. At the end of a 24 hour incubation at 37°C in an atmosphere of 5% CO_2 in air, ^{3}H-TdR (1 μCi/well) was added, the plates pulsed for 4 hours, the cells harvested and ^{3}H-TdR uptake measured using a scintillation counter.

IL1 bioassay

IL1 was quantified with a variation of an assay (Gearing *et al.*, 1987) based on the ability of IL1 to stimulate production of IL2 from a cell line which cannot incorporate ^{3}H-TdR (tk^-, EL4-6.1 cell line, known as NOB-1). Coculture of NOB-1 cells with IL2- responsive, tk^+, CTLL cells, that incorporate ^{3}H-TdR, allows IL1 to be assessed in terms of its stimulation of IL2 production by the NOB-1 cells. Reproducible assays required the NOB-1 cells to be harvested from log-phase cultures at densities of between 0.3-1 x 10^6/mL and CTLL cells to be harvested at densities between 10^5 - 10^6/mL. Briefly, 96-well microcultures were set up containing 100 μl of macrophage supernatants, or recombinant human IL1α standards, and 50 μL each of washed NOB-1 (5 x 10^4/well) and CTLL (10^4/well), with a final FBS concentration of 7.5% (vol/vol). A series of control wells contained only CTLL cells. The plate was incubated for 22-24 hours at 37°C in an atmosphere of 5% CO_2 in air, then pulsed for 5 hours with 1 μCi/well ^{3}H-TdR. The cells were harvested and cell associated ^{3}H-TdR uptake measured using a scintillation counter. The sensitivity of this assay is approximately 0.4 pg/mL pure recombinant *E. coli*-derived human IL1α. CSF-1, GM-CSF, G-CSF, IL3, TNFα and LPS did not stimulate NOB-1/CTLL ^{3}H-TdR uptake. IFNγ, at concentrations of greater than 100 U/mL slightly stimulated ^{3}H-TdR uptake from cocultures while IL4 (and IL2) directly stimulated CTLL growth, at levels >100 U/mL.

TNF bioassay

The assay, which measures the relative capacity of TNF to restrict the number of viable cells in cultures of the newly isolated TNF-sensitive fibroblastoid cell line L929-8, was performed as previously described (see Chapter IV; Branch *et al.*, 1991). Briefly, L929-8 cells were added to wells of a 96-well flat-bottom microtiter plate [5×10^4 /well in 50 μl of medium consisting of IMDM containing 15% (vol/vol) FBS plus 2 μg/mL Actinomycin D (Boehringer Mannheim, Laval, Quebec)], and preincubated for 2 hours at 37°C in 5% CO_2 in air. Fifty μL of macrophage supernatant, or recombinant murine TNFα standards, were then added in triplicate or quadruplicate to the wells of the microtiter dish, the cells cultured at 40°C for 20 hours, then pulsed for 2 hours with a 0.05% (wt/vol) solution of the dye neutral red. After washing once with 200 μL warm PBS, the color was developed using a solution of 0.05M NaH_2PO_4 in 50% (vol/vol) ethanol and the optical density determined on an automated microplate reader (sample wavelength 570 nm, reference 630 nm) as previously described. The lower limit of assay sensitivity is approximately 200 fg/mL using pure recombinant murine TNFα. This bioassay also detects TNFβ, but is not affected by other known cytokines.

IL6 bioassay

IL6 was measured as previously described (Lansdorp *et al.*, 1986) with minor modification. Briefly, macrophage culture supernatants or recombinant human IL6 were diluted in IMDM containing 15% (vol/vol) FBS and 50 μl added to wells of a 96-well microtiter plate containing 10^3 log-phase growing B9 hybridoma cells in 50 μL of the same medium. The plate was incubated for 3 days at 37°C in an atmosphere of 5% CO_2 in air, then pulsed for 4 hours with 1 μCi/well ^{3}H-TdR, harvested and the cell-associated ^{3}H-TdR measured using a scintillation counter.

CSF-1 radioreceptor assay (RRA)

The CSF-1 RRA was carried out as previously described (Das *et al*, 1980) with modification. Briefly, log-phase growing cultures of P388D1 cells (viability >95%) were collected and washed twice in ice-cold PBS, resuspended at 2 x 10^6/mL in IMB (IMDM lacking bicarbonate and adjusted to 280 mOsm and pH 7.35) and kept on ice. The assay was performed on wet-ice in 96-well flat-bottom microtiter plates. Sample or standards were added in duplicate or triplicate at 50 μL/well with control wells having either diluent alone (to determine maximum ^{125}I-CSF-1 bound) or 2 nM nonradioactive stage 1 CSF-1 (to determine nonspecific binding). Freshly harvested and washed P388D1 were added to each well in 50 μL and incubated for 2 hours prior to the addition of ^{125}I-CSF-1 (50 μl containing 5 x 10^4 cpm/well). After an additional 1 hour incubation, 100 μl aliquots from each well were layered over 200 μl ice-cold FBS in 500 μl polyethylene microfuge tubes. The tubes were centrifuged (10,000 x g, 2 minutes) and rapidly frozen in liquid nitrogen. The tips of the tubes containing the cell pellet were cut off and both cell-bound (pellet) and free radioactivity (supernatant) determined in a gamma counter. The extent of competition of an unknown sample was expressed as the unitless ratio, bound/free (B/F). The amount of competing CSF-1 in the sample was obtained from simultaneously derived standard curves of B/F versus CSF-1 concentration. Standard CSF-1 preparations were obtained from Dr. E.R. Stanley with 1 unit equal to 0.44 fmol (Stanley and Guilbert, 1981). Final values are calculated from the standard curve and expressed as units of CSF-1/mL.

Neutralizing antibodies

Neutralizing rabbit anti-murine GM-CSF was a gift from Dr. J.F. DeLamarter, Biogen, Geneva, Switzerland. Neutralizing goat anti-murine CSF-1 (lot R3A'84) and preimmune normal goat serum were gifts from Dr. E.R. Stanley, New York, NY. In experiments to determine the effect of anti-GM-CSF antibody on CSF-1-stimulated macrophage proliferation, the anti-GM-CSF or normal rabbit serum (NRS) control were added directly to the wells of 96-well microtiter plates, at the initiation of the experiment without preincubation, to give a final antibody or NRS control concentration of 0.5 to 1% (vol/vol). Experiments to determine the effect of anti-CSF-1 antibody on GM-CSF-stimulated macrophage proliferation were carried out in a similar fashion as described above for anti-GM-CSF. The final concentration of anti-CSF-1 or preimmune goat serum was 0.5%.

RESULTS

Cytokine effects on macrophage proliferation

Whether CSF-1 is the major mitogenic agent stimulating the proliferation of mouse macrophages and whether it acts alone has not been thoroughly examined (see Chapter I). In this section, results are presented using various cytokines and other agents to determine their effect alone or in combination with CSF-1 and/or GM-CSF or IL-3 on the proliferative response of well-defined populations of growth-competent mouse macrophages. The agents examined were: CSF-1, G-CSF, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-6, Epo, LIF, TGFβ, IFNγ, NGF, TNFα, TNFβ, PGE_2, and LPS. The rationale for selecting these particular agents is discussed in Chapter I (see Rationale for thesis).

Appendix 1a - The effect of G-CSF on CSF-1-stimulated macrophage proliferation

This figure shows CSF-1 to stimulate the proliferation of both BMM and S1 cells and that G-CSF alone has no effect on macrophage proliferation. However, when G-CSF is combined with CSF-1, the CSF-1-stimulated proliferative response is inhibited by approximately 40%. This inhibitory effect was reproduced in two additional experiments and was not seen with GM-CSF-stimulated macrophages.

Appendix 1b - The effect of GM-CSF on CSF-1-stimulated macrophage proliferation

This figure indicates that the cytokine GM-CSF can stimulate the proliferation of mouse macrophages. However, when combined with suboptimal levels of CSF-1, GM-CSF has opposing effects on the proliferative response depending on the macrophage population. When using primary BMM (A, top), GM-CSF over a wide concentration range (250 ng/mL to 2.5 ng/mL) appears inhibitory for the CSF-1-mediated proliferative signal. However, GM-CSF only appears to inhibit the CSF-1 portion of the mitogenic signal. In contrast, the S1 cell line shows strong synergy of CSF-1 with GM-CSF over the same concentration range (B, bottom). Thus, GM-CSF may be inhibitory for the CSF-1-mediated proliferation of BMM.

Appendix 1c - The effect of GM-CSF on CSF-1-stimulated macrophage proliferation

This figure indicates a confirmation of the results from the previous figure (see Appendix 1b). It shows results of titration studies using GM-CSF alone or GM-CSF combined with a fixed level of CSF-1 (taking care to insure that optimal, plateau levels of CSF-1 were maintained throughout the culture period) on the proliferation of C3H/HeJ BMM. The results clearly show GM-CSF to inhibit the CSF-1 response at all concentrations where GM-CSF alone can stimulate the proliferation of BMM; down to approximately 78 pg/mL in this experiment. Complete inhibition of the CSF-1-mediated proliferative response is attained at relatively high GM-CSF concentrations (>1.25 ng/mL). Thus, GM-CSF is a strong inhibitor of the CSF-1-mediated mitogenic signal, but still sends its own mitogenic signal.

Appendix 1d - Cytokine effects on CSF-1-stimulated mouse macrophage proliferation

This table indicates the effect on CSF-1-stimulated mouse macrophage proliferation of IL-6, IL-4, IL-2, Epo, LIF, TGFβ, LPS, and IFNγ. Alone, none of these agents stimulated macrophage proliferation. Differences in the effects of these agents when combined with maximally stimulatory levels of CSF-1 on the proliferation of mouse macrophages was evident. When using the S1 macrophage cell line, IL-2, IL-4, Epo and LIF appeared to have little effect on the CSF-1-mediated proliferation of these cells. In contrast, IL-6 and IFNγ may cooperate with CSF-1 to increase the proliferative response, while TGFβ and LPS appear to strongly down-modulate the CSF-1 response.

Results with BMM were similar, regardless of whether HeJ or HeN BMM were used, and differed from those results using S1 cells. IL-2, Epo and LIF had little affect on CSF-1-stimulated proliferation of BMM, and none of the agents examined showed additive or synergistic activity with CSF-1-stimulated BMM. On the contrary, IL-4, TGFβ, IFNγ and LPS all appeared to strongly down-regulate the CSF-1-mediated mitogenic signal while IL-6 showed less ability to down-modulate the CSF-1 response. It can be concluded from these results that the S1 cell line responses are very different from BMM responses. Furthermore, using BMM, IL-6, IL-4, TGFβ, IFNγ and LPS may negatively regulate the proliferation of these cells.

Appendix 1e - Cytokine effects on GM-CSF-stimulated mouse macrophage proliferation

This table indicates the effect on GM-CSF-stimulated mouse macrophage proliferation of IL-6, IL-2, Epo, LIF, TGFβ and IFNγ. Alone, none of these agents stimulated macrophage proliferation. Using S1 cells or BMM, no clear effect on GM-CSF-stimulated proliferation was seen using IL-6, IL-2 or LIF. Interestingly, Epo appeared to be inhibitory for GM-CSF-stimulated proliferation when using HeJ-derived macrophage populations but not when using HeN BMM. Also, TGFβ strongly down-regulated GM-CSF-stimulated proliferation of the S1 macrophage cell line but had no effect on BMM. However, all macrophage populations were strongly inhibited when IFNγ was combined with GM-CSF. Thus, exogenously added cytokines can have different effects on the proliferation of mouse macrophages depending on the stimulus (this appendix and Appendix 1d). Also, the results in this set of experiments using Epo are intriguing, however, only IFNγ has a clear effect (inhibitory) on GM-CSF-stimulated macrophage proliferation.

Appendix 1f - Effect of IL-1α on the proliferation of mouse macrophages

This figure shows that IL-1α (or TNFα) alone has no effect on macrophage proliferation. When IL-1α is combined with GM-CSF there is also no effect of IL-1α on the GM-CSF-stimulated proliferation of mouse macrophages. In contrast, when IL-1α is combined with CSF-1, a synergistic effect is seen on the proliferation of the S1 macrophage cell line while no effect on the CSF-1-mediated proliferation of BMM is seen. Again, these results indicate the differences in responsiveness of S1 cells compared to BMM. Moreover, these results suggest that S1 cells may be more responsive to combinations of cytokines than BMM, possibly this is related to the transformed nature of these cells. Also, the results in this figure indicate that IL-1α, unlike TNFα (see Chapter III), does not cooperate with CSF-1 on BMM.

Appendix 1g - Effect of lymphotoxin (TNFβ) on the proliferation of mouse macrophages

This figure shows the synergistic activity of lymphotoxin (LT; TNFβ) on the CSF-1-stimulated proliferative response using C3H/HeJ-derived macrophages and C3H/HeN BMM. A synergistic response is seen using all macrophage populations. These results support the contention that, of the cytokines yet examined, only the TNFs can cooperate with CSF-1, in a more than additive fashion, i.e., synergistic, to increase the maximal mitogenic signal provided by CSF-1 alone.

Appendix 1h - Effect of nerve growth factor on the proliferation of mouse macrophages

This table shows that 2.5s or 7s nerve growth factor (NGF) has no effect on the proliferation of mouse macrophages when used alone or in combination with plateau levels of CSF-1.

Appendix 1i - Effect of prostaglandin E_2 on the mitogenic stimulation of mouse macrophages

This table shows the effect of prostaglandin E_2 (PGE_2) on S1 macrophage proliferation during stimulation with CSF-1, GM-CSF and combinations of CSF-1 plus the synergistic agents GM-CSF, IL-3 or IL-1α. PGE_2 is inhibitory for all proliferative responses over a wide concentration range (10^{-5}M to 10^{-12}M). At concentrations of $> 10^{-6}$M, PGE_2 virtually shuts down any proliferative responses. However, the synergy effects are still evident, although much less responsiveness is seen than without PGE_2. These results suggest that PGE_2 is a powerful down-regulatory agent for macrophage proliferation, at least when using the S1 macrophage cell line.

Appendix 1j - Effect of prostaglandin E_2 on the CSF-1- and TNFα-mediated synergistic response using the S1 macrophage cell line

This figure illustrates the strong inhibitory effect of PGE_2 on the CSF-1 response and the CSF-1 plus TNFα synergistic response. At PGE_2 concentrations of 10^{-6}M, the CSF-1-mediated proliferative response is virtually shut down, however, synergy between TNFα and CSF-1 is still evident. At 10^{-10} M, PGE_2 inhibits approximately 50% of the response. These results argue that since the CSF-1/TNFα synergy response is inhibitable by PGE_2 using the S1 cells, that this effect will likely occur when using BMM (the rationale being that CSF-1 also synergizes with TNFα on BMM). However, BMM were not directly examined for the effect of PGE_2 on the proliferation of these cells.

Cytokine effects on CSF-1 binding to the CSF-1 receptor

The CSF-1 receptor can be down-modulated by at least three different mechanisms (see Chapter I), including a transient down-modulation induced by unrelated cytokines. This "trans-acting" effect has been suggested to be responsible for synergistic stimulatory effects observed when CSF-1 is combined with these trans-acting agents, however, the mechanism has never been elucidated. Moreover, a thorough examination of agents which can induce the down-regulation of CSF-1 binding to its receptor has not been reported. This section of the appendix examines many unrelated molecules for their ability to inhibit the binding of CSF-1 to its receptor.

Appendix 1k - Down-modulation of ^{125}I-CSF-1 binding to CSF-1 receptors

This table indicates the effect of various cytokines, proteins, and other agents on the binding of CSF-1 to its receptor. In general, all agents examined were able to down-regulate the binding of CSF-1 to its receptor. The ranking of cytokines for their ability to down-regulate CSF-1 binding to its receptor was CSF-1 > TNFα > IL-3 = GM-CSF > IL-1α = IFNγ > G-CSF. By far, CSF-1 and TNFα were the cytokines having the strongest down-modulating effect (>85% at 0.5 to 1 hour of exposure). In general, the down-regulatory effect was transient with normal binding of CSF-1 to its receptor returning by 24 hours of culture. In addition, interesting results were obtained with albumin. Using BMM, but not the S1 macrophage cell line, albumin was a strong down-regulatory agent for CSF-1 binding to its receptor. Other agents including transferrin, insulin, progesterone, estrogen and PGE$_2$ were also able to slightly modulate the binding of CSF-1 to its receptor. However, human IL-3 which does not bind to mouse IL-3 receptors also could slightly down-modulate the binding of CSF-1 to its receptor. With so many different agents capable of modulating the binding of CSF-1 to its receptor (some of these agents synergize with CSF-1, such as TNFα, while others, such as GM-CSF and IFNγ, are strong inhibitors of CSF-1 proliferation), the significance of the down-regulatory effect of these agents on the binding of CSF-1 to its receptor becomes unclear.

Production of endogenous cytokines from mouse macrophages

A number of cytokines have been reported to be produced by macrophages, including CSF-1, GM-CSF, G-CSF, IL-3, IL-1, IL-6, and TNFα (see Chapter I). Since exogenously added CSF-1, GM-CSF, IL-3 and TNFα can positively regulate macrophage proliferation and GM-CSF, G-CSF, and IL-6 may negatively regulate macrophage proliferation (see Appendix 1a-1e), and all of these cytokines can be produced by macrophages, a potential for autocrine networks exist. The following studies were to determine the endogenous cytokines which can be produced by the macrophage populations used in this thesis research.

Appendix 1l - Production CSF-1 from mouse macrophages

This table indicates the endogenous levels of CSF-1 which are constitutively produced or inducible from mouse macrophages detectable by RRA. The level of constitutive CSF-1 production was approximately 100 U/mL using HeJ-derived macrophage populations. GM-CSF, TNFα and IL-1α were all able to increase the basal level of CSF-1. IL-1α appeared to be the most effective, capable of increasing the levels of CSF-1 approximately 3-fold above basal levels. G-CSF had little effect on CSF-1 levels. Preliminary results were obtained with other agents or combinations of cytokines which indicate that CSF-1 production may be regulated by many agents. Thus, low levels of CSF-1 are constitutively produced or inducible from mouse macrophages.

Appendix 1m - Production of GM-CSF from mouse macrophages

This table indicates that GM-CSF production from mouse macrophages is inducible by CSF-1 (and possibly IFNγ) stimulation, but not by TNFα or IL-1α. Levels of neutralizable GM-CSF detectable after CSF-1 stimulation were 1.6- to 2.7-fold higher than basal levels. Thus, CSF-1 can induce the low level production of endogenous GM-CSF under appropriate conditions.

Appendix 1n - GM-CSF-stimulated production of endogenous cytokines from mouse macrophages

This table indicates that GM-CSF stimulation of mouse macrophages can induce the endogenous production of CSF-1, IL-1 and IL-6. In contrast, GM-CSF decreases the levels of TNFα which are produced constitutively in this culture system.

Appendix 1o - Production of IL-1 from mouse macrophages

This table shows results using culture supernatant for the detection of IL-1 produced by mouse macrophages after stimulation with various cytokines for 72 hours. When using GM-CSF, IL-1 appears to be inducible (the increased activity compared to medium control in the NOB-1 assay is neutralized with a pool of anti-mouse IL-1α and β antibodies) using BMM but not when using S1 cells. Furthermore, the combination of CSF-1 plus GM-CSF appears to induce even higher levels of IL-1, however, not all of the activity is neutralizable. Indeed, CSF-1 greatly increases the ^{3}H-TdR uptake of the NOB-1 cells, however, none of this activity is neutralizable with the pool of anti-mouse IL-1 antibodies. This activity may be a new activity, unrelated to IL-1. TNFα appears to be a strong inducing agent for the endogenous production of IL-1 from the S1 macrophage cell line but alone, TNFα does not induce any IL-1α from BMM.

Appendix 1p - Production of IL-1 from mouse macrophages

This table shows results using culture supernatant for the detection of IL-1 produced by mouse macrophage after stimulation with various cytokines for 24 hours. Within this time period, and controlling for DNA content, there appears to be no induction of IL-1 from BMM using any cytokine or combination of cytokines. However, when using the S1 cell line, TNFα is able to induce high levels of IL-1. These results are in conflict with the previous table (see Appendix 1o) but may indicate that the kinetics of IL-1 release from mouse macrophages is longer than 24 hours. Thus, these results may indicate that IL-1 levels associated with the cell may be more informative.

Appendix 1q - Production of IL-6 from mouse macrophages

This table indicates that IL-1α can induce relatively high levels of IL-6 from mouse macrophages. In addition to the results with IL-1α, GM-CSF has also been shown to induce endogenous production of IL-6 from mouse macrophages (see Appendix 1n). CSF-1 does not induce the production of IL-6 from BMM or S1 cells (data not shown).

Appendix 1r - Effect of blocking endogenously produced GM-CSF on the CSF-1-stimulated proliferation of mouse macrophages

Since CSF-1 can induce the production of GM-CSF from mouse macrophages (see Appendix 1m), this figure examines the relative role of this endogenously produced GM-CSF on the CSF-1-mediated proliferative response. Neutralizing anti-mouse GM-CSF antibody had little effect on the CSF-1-stimulated proliferation of BMM. Thus, endogenously produced GM-CSF appears to have no effect on CSF-1-stimulated primary macrophages. In contrast, anti-mouse GM-CSF strongly down-regulates the CSF-1-mediated proliferation of the S1 macrophage cell line. This result suggests that endogenously produced GM-CSF can act in an autocrine manner to increase the CSF-1-mediated proliferative response of this transformed macrophage cell line. However, secreted GM-CSF appears to have no role for the proliferation of normal macrophages.

Appendix 1s - Effect of blocking endogenously produced CSF-1 on the GM-CSF-stimulated proliferation of mouse macrophages

Since constitutive CSF-1 levels are detectable with our macrophage populations and GM-CSF can increase the level of CSF-1 approximately 2- to 5-fold above basal levels (see Appendix 1l), this figure examines the relative role of this endogenously produced CSF-1 on the GM-CSF-mediated proliferative response. Neutralizing anti-mouse CSF-1 antibody had little effect on the GM-CSF-stimulated proliferation of BMM. In contrast, anti-mouse CSF-1 allowed for enhanced proliferation with the S1 macrophage cell line stimulated with GM-CSF. This result suggests that endogenously produced CSF-1 may act as an autocrine inhibitory molecule for GM-CSF-stimulated proliferation of transformed S1 cells. However, secreted CSF-1 appears to have no role for the proliferation of normal macrophages.

In conclusion, low levels of CSF-1 are detectable from BMM. However, experiments using neutralizing anti-CSF-1 showed no effect of inhibiting this low level of endogenously produced CSF-1 on BMM proliferation. Likewise, anti-GM-CSF shows no effect on BMM proliferation. Thus, autocrine/juxtacrine/paracrine mechanisms involving endogenously produced CSF-1 or GM-CSF are not found influencing BMM proliferation and may not be operative. However, intracrine mechanisms cannot be ruled out. Endogenous production of TNFα may influence the proliferative response of BMM (see Chapters V and VI). Endogenous production of IL-6 may also affect the proliferation of BMM (see Appendix 1d). Further studies are necessary to better define the role of all of these cytokines as endogenous modulators of normal mouse macrophage proliferation.

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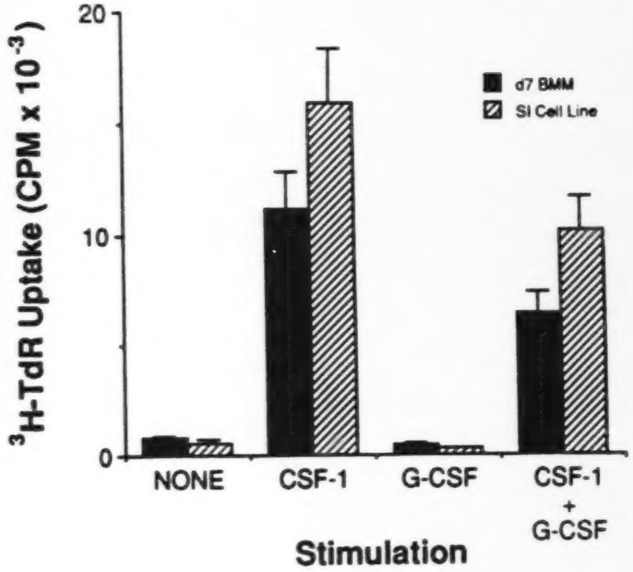
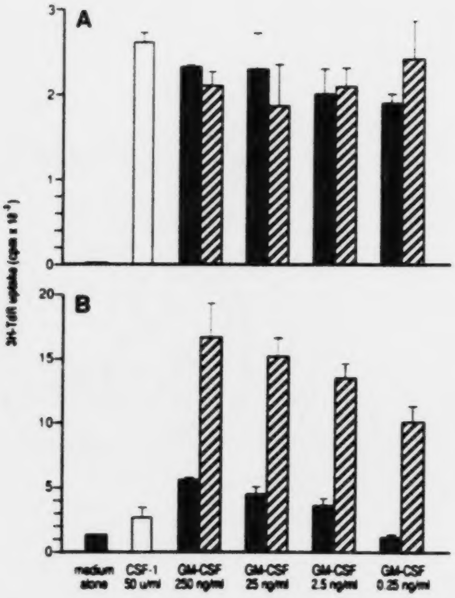


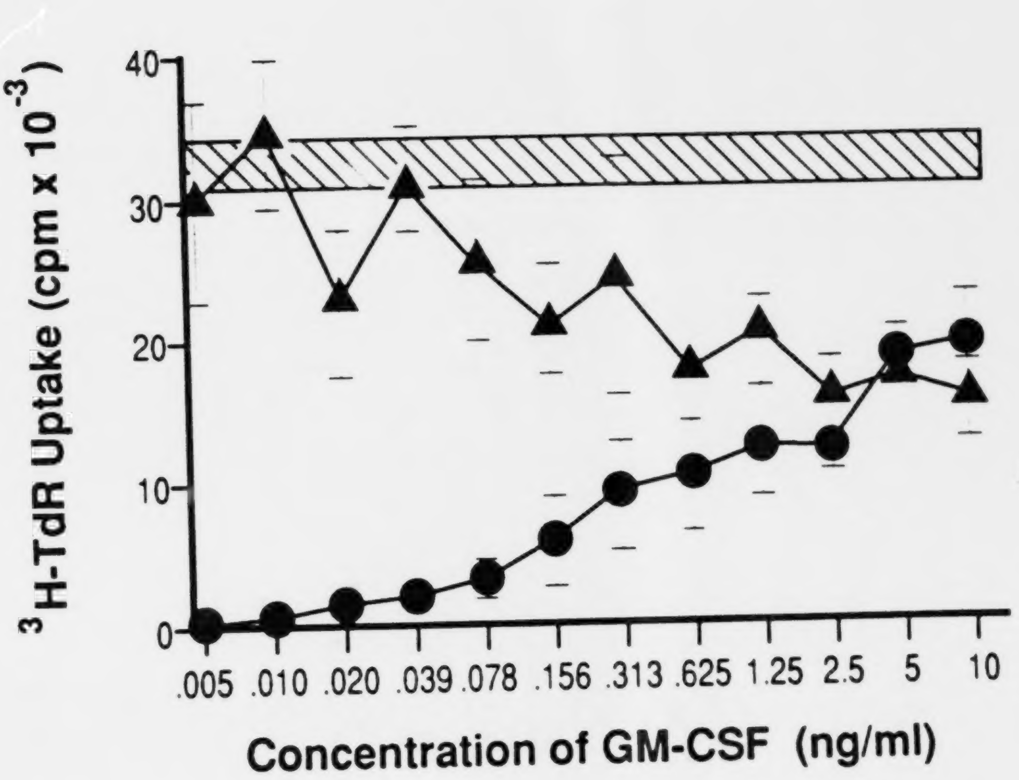
Appendix 1a

The effect of G-CSF on CSF-1-stimulated macrophage proliferation. 10^3 primary BMM (■) or the S1 macrophage cell line (▨) were stimulated with CSF-1 (5 x 10^3 U/mL) , G-CSF (1250 U/mL) or both CSF-1 and G-CSF for 3 days, the ^{3}H-TdR added, the cells harvested and the amount of ^{3}H-TdR incorporation measured with a scintillation counter. In this representative experiment (1 of 3) G-CSF inhibited the CSF-1-mediated proliferation of BMM by 42% and S1 cells by 35%.



Appendix 1b

The proliferative response of C3H/HeJ d7 BMM (panel A) or the v-*myc*-transformed S1 macrophage cell line (panel B) to GM-CSF over the concentration range 250 ng/mL down to 0.25 ng/mL with or without a fixed suboptimal level of CSF-1 (50 U/mL). The means $\underline{+}$ standard deviation of triplicate determinations are shown. Striped boxes represent ^{3}H-TdR uptake using medium alone; open boxes represent stimulation by 50 U/mL CSF-1 alone; solid boxes represent stimulation by the indicated concentration of GM-CSF alone; cross-hatched boxes represent costimulation by GM-CSF and CSF-1.



Appendix 1c

Effect of GM-CSF alone (●) on the proliferative response of C3H/HeJ d7BMM using doubling dilutions, starting at 10 ng/mL. Effect of GM-CSF in the presence of a fixed, maximally stimulatory (plateau) level of CSF-1 (▲). Each point represents the mean ^{3}H-TdR incorporation ± standard deviation after stimulation with GM-CSF over 3 days. The hatched box represents the mean ^{3}H-TdR incorporation ± standard deviation for the CSF-1 alone (10,000 U/mL).

Appendix 1d

Cytokine effects on CSF-1-stimulated mouse macrophage proliferation

Cytokine	n[+]	S1 Cell Line	d7HeJ BMM	d7HeN BMM
IL-6 (1/1000 cos)	4	1.27 ± 0.39[*]	0.87 ± 0.21	0.81 ± 0.24
IL-4 (50 ng/mL)	1	0.89	0.49	NT
IL-2 (17.5 U/mL)	1	0.87	1.08	0.86
Epo (6 U/mL)	2	1.04; 1.34	0.87; 1.09	0.80 (n=1)
LIF (1250 U/mL)	1	0.91	0.97	1.05
TGFβ (5 ng/mL)	1	0.24	0.58	0.44
LPS (5 μg/mL)	1	0.12	0.11	0.004
IFNτ (100 U/mL)	2	1.47 (n=1)	0.53; 0.30	0.46; 0.32

None of the cytokines alone stimulated macrophage proliferation

[+] number of independant experiments done in triplicate

[*] ratio of ^{3}H-TdR uptake = $\frac{\text{CSF-1 + Cytokine}}{\text{CSF-1}}$

Human recombinant interleukin 6 (IL-6) and erythropoietin (Epo) were from Genetics Institute, Boston, MA; murine, pure recombinant IL-4 was from Immunex Corp., Seattle, WA.; human, pure recombinant IL-2 was a gift from Dr. V. Paetkau, Department of Biochemistry, University of Alberta; human, pure leukemia inhibitory factor (LIF) was a gift from Dr. N. Gough, Melbourne, Australia; porcine recombinant transforming growth factor-β (TGFβ) was obtained from R & D Systems, Minneapolis, MN; Lipopolysaccharide (LPS) was obtained from Sigma Chemical, St. Louis, MO; murine, pure recombinant interferon-τ (IFNτ) was a gift from Dr. C. Stock, American Cancer Society, New York, NY.

Appendix 1e

Cytokine effects on GM-CSF-stimulated mouse macrophage proliferation

Cytokine	n$^+$	S1 Cell Line	d7HeJ BMM	d7HeN BMM
IL-6 (1/1000 cos)	2	1.07; 0.96$^{\bullet}$	0.82; 0.90	0.57; 1.15
IL-2 (17.5 U/mL)	1	1.16	0.94	1.00
Epo (6 U/mL)	1	0.65	0.68	1.05
LIF (1250 U/mL)	1	1.75	0.93	0.95
TGFβ (5 ng/mL)	1	0.29	0.96	1.18
IFNγ (100 U/mL)	2	0.48 (n=1)	0.48; 0.51	0.39; 0.26

None of the cytokines alone stimulated macrophage proliferation.

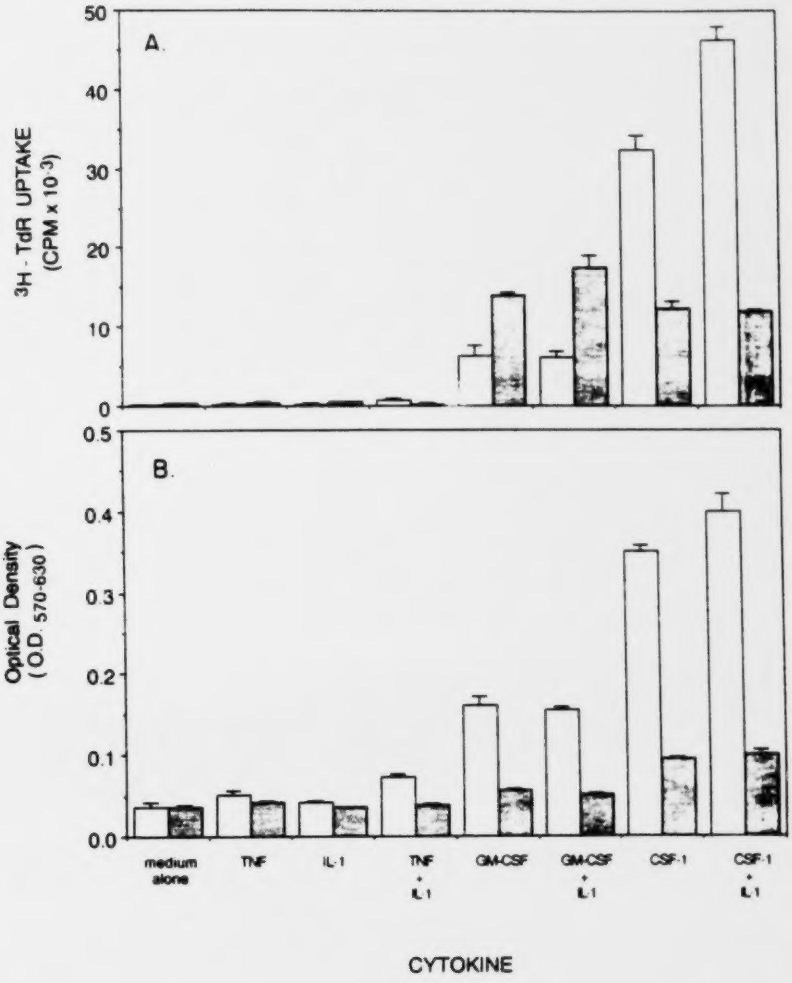
$^+$ number of independent experiments done in triplicate

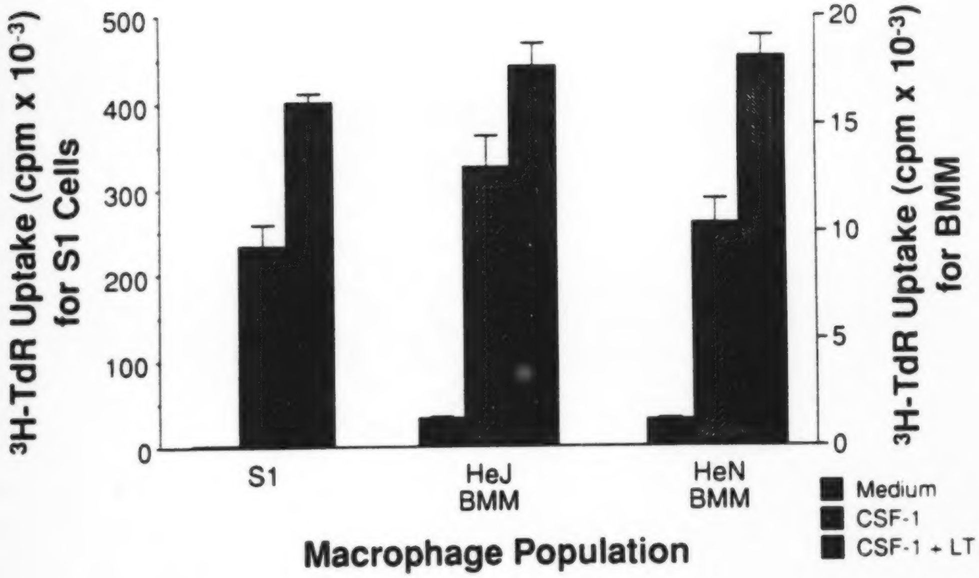
$^{\bullet}$ ratio of ^{3}H-TdR uptake $= \dfrac{\text{GM-CSF + Cytokine}}{\text{GM-CSF}}$

Human recombinant interleukin 6 (IL-6) and erythropoietin (Epo) were from Genetics Institute, Boston, MA; human, pure recombinant IL-2 was a gift from Dr. V. Paetkau, Department of Biochemistry, University of Alberta; human, pure leukemia inhibitory factor (LIF) was a gift from Dr. N. Gough, Melbourne, Australia; porcine recombinant transforming growth factor-β (TGFβ) was obtained from R & D Systems, Minneapolis, MN; murine, pure recombinant interferon-γ (IFNγ) was a gift from Dr. C. Stock, American Cancer Society, New York, NY.

Appendix 1f

Proliferation of the murine C3H/HeJ S1 cell line (open) and d7 C3H/HeJ BMM (hatched) stimulated by medium alone, TNFα, IL-1α, GM-CSF, CSF-1 or mixtures of these cytokines. **A.** Proliferation measured using incorporation of ^{3}H-TdR into DNA after stimulation of 10^3 cells for 3 days in culture by TNFα (155 ng/mL); IL-1α (1250 U/mL); GM-CSF (5 ng/mL); and recombinant human CSF-1 (16,500 U/mL). **B.** Total viable cell content as a function of MTT reduction after stimulation of 10^3 cells for 3 days in culture by TNFα (1550 ng/mL); IL-1α (1250 U/mL); GM-CSF (5 ng/mL); and recombinant human CSF-1 (16,500 U/mL). The mean and standard deviations of three replicates are shown.


A.
B.
3H - TdR UPTAKE
(CPM x 10-3)
0
10
20
30
40
50
Optical Density
(O.D. 570-630)
0.0
0.1
0.2
0.3
0.4
0.5
medium alone
TNF
IL-1
TNF + IL-1
GM-CSF
GM-CSF + IL-1
CSF-1
CSF-1 + IL-1
CYTOKINE



Appendix 1g

Macrophages (10^4/mL) were cultured in medium alone or in a fixed amount of CSF-1 (50 ng/mL; Genetics Institute, Boston, MA) with or without LT (TNFβ; 10 ng/mL; Genentech, South San Francisco, CA). After 3 days, proliferation was assessed after ^{3}H-TdR uptake over 4 hours and measured in a scintillation counter. For the indicated macrophage population, solid boxes indicate results using medium without CSF-1 or LT; hatched boxes indicate CSF-1 alone; speckled boxes indicate CSF-1 + LT. Results are presented as the means ± SD of quadruplicate determinations. LT did not stimulate macrophage proliferation on its own.

Appendix 1h

Effect of nerve growth factor (NGF) on mouse macrophage proliferation

5/10.14 Macrophages Stimulated with:	CPM ± SD		
Medium Alone	1,039	±	757
CSF-1 16/5 (0.5%)	71,664	±	7,563
CSF-1 + NGF 2.5s (1 μg/mL)	67,965	±	405
CSF-1 + NGF 7s (1 μg/mL)	67,927	±	6,043
NGF 2.5s alone	2,745	±	81
NGF 7s alone	1,428	±	718

NGF was a gift from Dr. R. Murphy, Department of Anatomy and Cell Biology, University of Alberta.

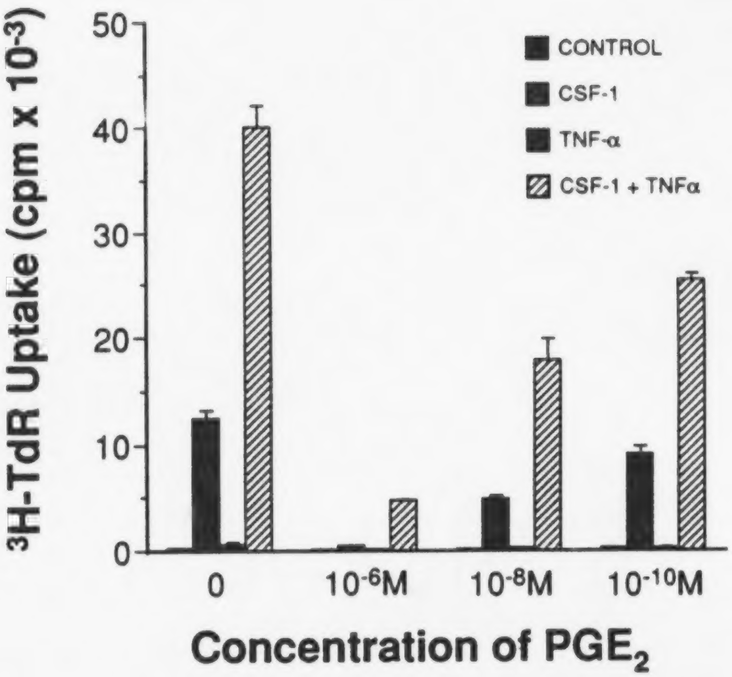
Appendix II

Effect of PGE_2 on the mitogenic stimulation of the mouse macrophage cell line S1 using CSF-1, GM-CSF, IL-3, IL-1 and combinations of these growth factors

					CSF-1 PLUS:		
[PGE_2]	CSF-1	GM-CSF	IL-3	IL-1	GM-CSF	IL-3	IL-1
0	15,615±3,163	11,099±332	2550±421	945±131	34,241±2,891	14,555±2,370	22,096±1,758
10^{-5}M	193±73	313±116	NT	153±81	1,381±86	NT	1,362±937
10^{-6}M	361±30	660±285	NT	206±85	2,744±178	490±137	2,788±66
10^{-8}M	4,619±988	5,551±336	NT	259±57	13,640±553	6,436±810	9,595±123
10^{-9}M	7,641±1,382	7,811±472	NT	520±42	19,251±994	NT	13,125±1,156
10^{-10}M	8,043±983	6,826±717	NT	769±590	20,382±2,243	NT	13,773±1,204
10^{-12}M	10,760±2,617	6,912±716	NT	625±266	17,475±1,702	NT	14,203±991

NT = not tested

PGE_2 = prostaglandin E_2 (Sigma Chemical, St. Louis, MO)



Appendix 1j

The S1 macrophage cell line was cultured for 3 days with the cytokines indicated in the absence or presence of varying concentrations of PGE_2 (10^{-6}M, 10^{-8}M, 10^{-10}M). Results are presented as the mean ± SD of triplicate determinations using ^{3}H-TdR uptake as readout.

Appendix 1k

Down-Modulation of ^{125}I-CSF-1 binding to CSF-1 receptors at 4°C

		% INHIBITION OF ^{125}I-CSF-1 BINDING			
		d7 BMM		S1 Cell Line	
Agent	Concentration Tested	0.5 to 1 hr. Exposure	24 hr. Exposure	0.5 to 1 hr. Exposure	24 hr. Exposure
AFP (95% pure)	160 ug/ml	8.1	4.5	0.9	39.5
ALB (from AFP purification)	160 ug/ml	53.4	0	32.8	19.8
Mouse ALB	200 ug/ml	78.7	9.1	0	NT
BSA (fatty acid free)	200 ug/ml	25.3	18.8	0	NT
BSA (fraction V)	200 ug/ml	15.5	38.7	0	NT
Transferrin	5 ug/ml	12.9	19.6	0	NT
Insulin*	10 ug/ml	21.7	15.5	0	42.9
Progesterone*	10 ug/ml	[illegible]7.1	18.9	12.6	28.6
Estrogen*	10 ug/ml	25.2	12.8	14.6	39.3
IL-1-alpha	1000 ug/ml	26.0	14.6	23.9	0
CSF-1	6600 u/ml	100	98.8	100	NT
TNF-alpha	1240 u/ml	86.3	0	90.0	0
GM-CSF	1.6 ng/ml	53.6	13.8	89.7	NT
Mu IL-3	1/50 COS sup.	60.4	0	83.3	0
G-CSF	1/100 G.I.	25.0	14.3	8.3	0
IFN-gamma	100 u/ml	23.9	13.9	41.0	NT
PGE_2	4×10^{-9}M	37.1	26.3	54.6	23.7
hIL-3	unk	10.3	11.9	21.9	21.9
* Alcohol Control		11.6	10.6	9.0	18.5

Nonspecific binding results were subtracted prior to calculating % inhibition of ^{125}I-CSF-1 binding.

Appendix 11
Constitutive and inducible CSF-1 production from mouse macrophages detected by specific radioreceptor assay

Macrophages Stimulated with	n	CSF-1 (U/mL) Detected in Supernatants from: S1 Cell Line	d7HEJ BMM	BAC1.2F5 (n=1)
Medium Alone	9	**112 ± 118** (0 to 307)	**103 ± 100** (0 to 203)	**<31.2**
GM-CSF	2	**258** (77; 438)	**500** (0; 1,000)	**81**
TNF-α	3	**260 ± 439** (0 to 1,042)	**267 ± 416** (10 to 1,000)	**0**
IL-1-α	3	**378 ± 351** (53 to 750)	**351 ± 348** (114 to 750)	**0**
G-CSF	4	**62 ± 111** (0 to 228)	**112 ± 160** (0 to 356)	**NT**
IFN-τ	1	**193**	**118**	**0**
PGE$_2$	2	**250** (0; 500)	**82** (0; 164)	**0**
LPS		**NT**	**NT**	**30**
GM-CSF + TNF	1	**1,213**	**1,212**	**NT**
GM-CSF + IL-1	1	**0**	**0**	**NT**
TNF + IL-1	2	**155** (104; 206)	**82** (0; 163)	**0**
TNF + G-CSF	1	**32**	**14**	**NT**
TNF + G-CSF + GM-CSF	1	**18**	**28**	**NT**

5 x 10^4 cells/mL using microtiter plates, cultured with or without indicated agent for 3 days, centrifuged, supernatant collected and assayed for CSF-1 by radioreceptor assay. Range is indicated in parenthesis.

Appendix 1m

Production of neutralizable GM-CSF from mouse macrophages

		Macrophage supernatant collected from:		
Cytokine used to stimulate:	n	d7 HeJ BMM	n	S1 Cell Line
CSF-1 (1250 U/mL)	4	**1.62 ± 0.51*** (1.10 to 2.28)	4	**2.73 ± 1.00** (1.58 to 3.97)
TNF-α (125 ng/mL)	6	**1.03 ± 0.82** (0 to 2.41)	7	**0.96 ± 0.42** (0.62 to 1.88)
IL-1α (125 U/mL)	2	**1.27** (1.17,1.37)	2	**0.83** (0.72,0.93)
IFNγ (100 U/mL)	1	**3.40**	1	**1.06**

* fold increase over basal levels (d7 BMM, 27 ± 5 pg/mL, n=4; S1 cell line, 31 ± 6 pg/mL, n=4) of neutralizable GM-CSF activity in macrophage supernatants collected after stimulation of 1-5 x 10^4 cells/mL with the indicated cytokines for 1 to 4 days. Numbers in parentheses represent the range of activity. Neutralizing anti-GM-CSF completely inhibits 1 ng/ml pure recombinant murine GM-CSF; it has no effect on G-CSF, IL-3, or Epo (LIF was not tested). Lower limit of GM-CSF detection using the DA-1 bioassay is approximately 20 pg/mL using recombinant murine GM-CSF (Immunex).

Appendix 1n

GM-CSF-stimulated cytokine production from mouse macrophages

	Culture supernatant[*] from :		
Cytokine Assayed	d7HeJ BMM	d7HeN BMM	S1 Cell Line
CSF-1	5.5[#]	NT	2.5
TNF	0.5	0.01	<0.01
IL-1	2.5	>10	<0.5[+]
IL-6[&]	197	NT	12

[*] Macrophage populations were incubated for 3 days in the presence of 25 to 50 ng/mL pure recombinant GM-CSF and the supernatants collected for testing (see Methods).

[#] Fold increase in cytokine activity above background (basal) levels (cells + medium alone).

[+] GM-CSF down-regulates a very low basal level.

[&] Absolute levels are similar because S1 basal levels are high.

Appendix 1o
Soluble IL-1 activity from unstimulated and stimulated murine macrophages detected after 72 hours of culture#

	NOB-1/CTLL Growth* stimulated by medium conditioned by:		
Macrophages stimulated by:	HeJ d7BMM	HeN d7BMM	S1 Cell Line
Unstimulated (medium alone)	**721 ± 534** (422 ± 530)	**0 ± 227** (0 ± 284)	**80 ± 27** (0 ± 4)
CSF-1	**5,228 ± 485** (6,017 ± 650)	**2,231 ± 1,091** (3,020 ± 128)	**0 ± 39** (53 ± 241)
GM-CSF	**1,800 ± 370** (0 ± 76)	**2,886 ± 1,318** (646 ± 233)	**93 ± 165** (843 ± 623)
TNF	**1,078 ± 747** (0 ± 79)	**241 ± 1,071** (0 ± 107)	**13,304 ± 552** (1,054 ± 354)
CSF-1 + GM-CSF	**5,425 ± 1,155** (1,799 ± 280)	**11,859 ± 558** (4,791 ± 656)	**0** (NT)
CSF-1 + TNF	**90 ± 412** (1,156 ± 281)	**0 ± 194** (NT)	**822 ± 45** (NT)
GM-CSF + TNF	**NT**	**NT**	**12,941 ± 1484** (1,277 ± 365)

Cells were plated at 5 x 10^4 cells/ml and cultured for 3 days in the presence of medium alone or individual cytokines or combinations of cytokines. The cells were centrifuged at 1000 x g and the supernatants removed for testing

* CPM with background subtracted using ^{3}H-TdR uptake after 24 h. Numbers in parenthesis represent results of neutralizing experiments using a pool of anti-murine IL-1a and IL-1β. Control values for supernatant collected from LPS stimulated RAW 264.7 were 14,737 ± 247 cpm without blocking antibodies and 153 ± 229 with blocking antibodies.

Appendix 1p

Soluble IL-1 activity from unstimulated and stimulated mouse macrophages detected after 24 hours of culture[#]

Macrophages stimulated by:	NOB-1/CTLL Growth[*] stimulated by medium conditioned by:		
	HeJ d7GMM	HeN d7BMM	S1 Cell Line
Unstimulated (medium alone)	2,830 ± 217 (22.5)	1,887 ± 402 (18.5)	3,330 ± 915 (70.4)
CSF-1	2,652 ± 329 (11.2)	2,340 ± 88 (15.7)	973 ± 261 (18.7)
GM-CSF	2,592 ± 596 (13.5)	2,062 ± 499 (14.9)	1,124 ± 326 (18.5)
TNF	2,826 ± 336 (22.4)	2,271 ± 255 (27.5)	26,152 ± 1,134 (404.2)
CSF-1 + GM-CSF	2,924 ± 333 (15.4)	5,100 ± 169 (30.4)	1,964 ± 676 (22.9)
CSF-1 + TNF	2,746 ± 306 (12.7)	2,636 ± 642 (16.8)	1,932 ± 614 (24.0)
GM-CSF + TNF	2,615 ± 624 (13.7)	2,157 ± 522 (15.7)	8,764 ± 1,667 (109.6)

[#] Primary cells were plated so that a final concentration of approximately 5 x 10^5 cells/ml would be reached by day 7 of culture; S1 cell line was plated at 5 x 10^5 cells/ml. After stimulation by medium alone, individual cytokines or mixture of cytokines, supernatant was harvested and centrifuged at 10,000 x g before testing.

[*] CPM using ^{3}H-TdR uptake after 24 h. Numbers in parenthesis represent cpm per ng of DNA content (see Methods)

Appendix 1q
Soluble IL-6 production from IL-1 stimulated mouse macrophages

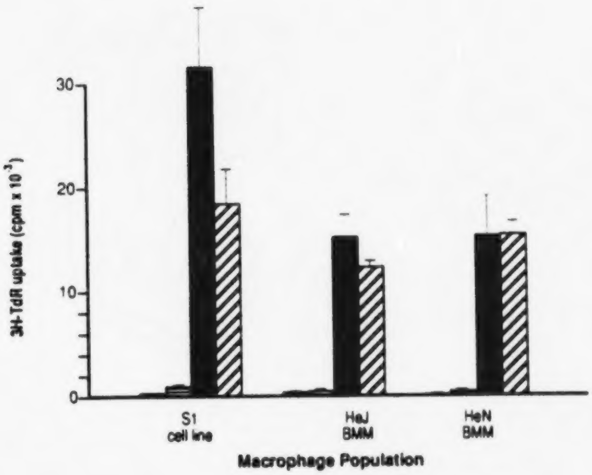
Macrophages stimulated by:	B9 Cell Growth[#] Stimulated by medium conditioned by: d7 HeJ BMM	S1 Cell Line
Unstimulated (medium alone)[*]	169 ± 29	3,123 ± 690
IL-1 (1250 U/mL)	2,479 ± 66	27,861 ± 3,185

[*] Control background (B9 cells plus medium) = 198 ± 61.

[#] IL-6 levels measured by B9 growth assay as described in the Methods. Data are the mean cpm ± SD of triplicate assays.

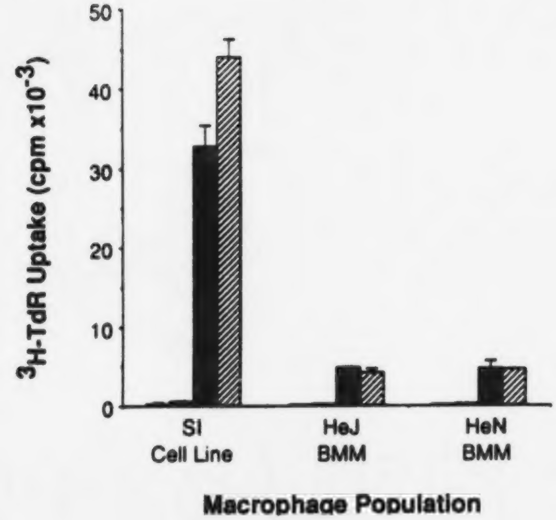
Appendix 1r

Effect of neutralizing rabbit anti-mouse GM-CSF on the CSF-1-stimulated proliferation of mouse macrophages.



Effect of neutralizing rabbit anti-mouse GM-CSF on the CSF-1-stimulated proliferation of murine macrophages. The anti-GM-CSF antibody was heat-inactivated at 56°C for 30 minutes prior to use with no loss of ability to neutralize pure recombinant mouse GM-CSF using a DA-1 bioassay (data not shown). Results for proliferation are presented as the mean ^{3}H-TdR uptake ± standard deviation. Medium (open boxes) or anti-GM-CSF (stripped boxes) alone gave the same, essentially background, results. Solid boxes show the proliferation of the indicated macrophage population to suboptimaimal concentrations of pure, human recombinant CSF-1 alone (12.5 ng/mL). Hatched boxes show the proliferation of the indicated macrophage populations to CSF-1 in the presence of 0.5% anti-mouse GM-CSF.

Appendix 1s
Effect of neutralizing goat anti-mouse CSF-1 on the GM-CSF-stimulated proliferation of mouse macrophages



Effect of neutralizing goat anti-mouse CSF-1 on the GM-CSF-stimulated proliferation of murine macrophages (10^4 cells/mL over 3 days). Results for proliferation are presented as the mean ^{3}H-TdR uptake ± standard deviation of triplicate or quadruplicate determinations. Medium (open boxes) or anti-CSF-1 (stripped boxes) alone gave essentially background results. Solid boxes show the proliferation over 3 days of the indicated macrophage population to optimally stimulatory (plateau) concentrations of GM-CSF (62.5 ng/mL). Hatched boxes show the proliferation over 3 days of the indicated population to GM-CSF in the presence of 0.5% anti-mouse CSF-1. Normal goat prebleed serum (0.5%) did not affect the assay (data not shown).

APPENDIX 2

ANTISENSE INHIBITION OF TUMOR NECROSIS FACTOR-ALPHA PROTEIN USING SYNTHETIC PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

INTRODUCTION

Cytokines are produced by many cell types in response to various stimuli and have important functions in cellular communication regulating cell growth, differentiation and biological function (1, 2). The expression of many cytokine genes appears to be controlled by other cytokines, thus, forming a complex network of interdependent regulation (3). The complexity of this system makes it difficult to isolate and analyze the regulation of one particular cytokine to assess its biological role. Although one could use neutralizing antibody to investigate the role of paracrine/juxtacrine/autocrine acting proteins, this would require the produced protein to be accessible to the effects of the neutralizing antibody. In situations where the protein can act within the cytosol or is not otherwise accessible to antibody, methods for directly inhibiting the production of proteins may be necessary.

Inhibition of gene expression using synthetic antisense oligodeoxynucleotides has been used to study the role of the proteins c-*myc* (4), c-*myb* (5), proliferating cell nuclear antigen (6) and the cytokine CSF-1 (7) in cell growth and differentiation. It is possible, therefore, to inhibit synthesis of a specific cytokine, using antisense oligodeoxynucleotides, in order to define the function of those cytokines whose production it regulates. Of special interest to us is the potential role endogenous cytokines may play in the autocrine modulation of cell growth and proliferation (8).

Previously (9), we have demonstrated a cooperative effect of TNFα with the macrophage growth factor CSF-1 on the proliferation of mouse macrophages. Moreover, TNFα is an inflammatory molecule produced by macrophages (10). Thus, endogenously produced TNFα may contribute to the observed proliferative response of mouse macrophages to CSF-1 or other macrophage growth factors such as GM-CSF. Therefore, we have examined the potential usefulness of synthetic phosphorothioate analogues of oligodeoxynucleotides in blocking translation of TNFα mRNA in mouse macrophages after stimulation with LPS or CSF-1. The sulfur-modified oligodeoxynucleotides (S-oligos) were chosen in this work due to their increased resistance to degradation by nucleases, compared with unmodified oligodeoxynucleotides (11), coupled with efficient hybridization to RNA and cell accessibility (11). These properties should allow more effective inhibition of gene expression than is possible with unmodified oligodeoxynucleotides (11).

In this communication we show that LPS- and CSF-1-stimulated TNFα protein production in macrophages can be specifically inhibited by S-oligos made antisense to TNFα mRNA. The degree of inhibition is dependent on the level of TNFα produced by the macrophages, which is, in turn, dependent on the population of macrophages used and the stimulus. This is the first report of successful blocking of TNFα protein production using synthetic antisense oligodeoxynucleotides.

MATERIALS AND METHODS

Cells

Primary, bone marrow-derived macrophages (BMM), prepared from the femurs of C3H/HeJ or C3H/HeN mice, using a series of short-term-culture selection steps at days 1, 3, 5, and 7, were obtained as previously described (9). Adherent primary cells at day 7 of culture (d7 BMM) were harvested and used in culture and immunoprecipitation assays. The procedure yields a population of 'young', growth factor-dependent macrophages of established homogeneity (9).

The growth factor-dependent C3H/HeJ macrophage cell line S1 was cloned from the v-*myc*-transformed 5/10-14 macrophage cell line as previously described (9). The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection, Rockville, MD. L929-8 mouse fibroblastoid cells were isolated from parent L929 cells and maintained as previously described (12).

Cytokines and antibodies

Pure, recombinant human CSF-1 (2.1 x 10^6 U/mL) was a gift of Genetics Institute, Boston, MA. Pure, recombinant mouse TNFα (1.2 x 10^7 U/mg) and neutralizing rabbit anti-mouse TNF, was obtained from Genentech, South San Francisco, CA. Lipopolysaccharide (LPS from *E. coli*, strain 026-B6) was from Sigma Chemical, St. Louis, MO.

TNF bioassay

The bioassay used has been previously described (12) and measures the relative capacity of TNF to restrict the number of viable cells in cultures of the newly isolated TNF-sensitive fibroblastoid cell line L929-8. Briefly, cells were cultured with sample, or TNFα standards, in the presence of 2 μg/mL actinomycin D, at 40°C in 5% CO_2 in air, over a 20 hour period. Remaining viable cells were estimated, colorimetrically, after being pulsed for 2 hours with a 0.05% (wt/vol) solution of the vital dye neutral red and color developed using a solution of 0.05M NaH_2PO_4 in 50% (vol/vol) ethanol. The optical density at 570 nm was read on an automated microplate reader as previously described (12). The lower limit of assay sensitivity is approximately 200 fg/mL using pure, recombinant mouse TNFα (12). The bioassay also detects TNFβ but is not affected by other cytokines (12), or by S-oligos (data not shown).

Phosphorothioate oligodeoxynucleotides.

Phosphorothioate oligodeoxynucleotides (S-oligos) were chosen for their effective hybridization for DNA:RNA heteroduplex formation, significant nuclease-resistance, solubility, and specific receptor-mediated cellular uptake (11). Based on the published cDNA sequence for mouse TNFα (13), an S-oligo having the sequence 5'-GGATCATGCTTTCTGTGCTCAT-3', which was complementary to the first 22 nucleotides of the sense strand of mouse TNFα mRNA, starting with nucleotide 144 of the initiation start site codon (AUG), was purchased from Synthecell Corporation, Rockville, MD. The FASTA system gene-banks search (14), indicated that this nucleotide sequence, when analyzed in the antisense direction, had a high degree of homology with various animal TNFα genes without significant homologies to other genes, when evaluated in either the sense or antisense direction. A number of base-matched 22-mer S-oligo nonsense sequences were evaluated, both in the sense and antisense direction, for significant homologies with other genes using FASTA (14). The nonsense sequence 5'-GTCAGGTCGTTACTCGTTTACT-3' was purchased from Synthecell. This sequence was base-matched to the antisense sequence and designed to have minimal homologies to any known genes. S-oligos were diluted in phosphate buffered saline (PBS) and kept at -70°C prior to use.

Immunoprecipitation

Immunoprecipitation was performed with slight modification for use with oligodeoxynucleotides (7). Briefly, 0.5 mL of 10^6 washed cells in serum-free medium lacking methionine and cysteine (RPMI-1640 Select-amine, GIBCO), were added to 35 mm tissue culture dishes. Antisense or nonsense S-oligos, or oligo diluent (PBS control), were immediately added to appropriate dishes. After incubation for 2 hours at 37°C in 5% CO_2 in air, dialyzed fetal bovine serum (FBS), which had been batch-adsorbed with polymyxin B-sepharose (Sigma) to remove any contaminating LPS, was added to a concentration of 5-10% (vol/vol). After one additional hour at 37°C and 5% CO_2, LPS or cytokines were added for 30 minutes followed with 100 μCi ^{35}S-methionine (Dupont Canada, Mississauga, ON; >1000 Ci/mmol) and 100 μCi ^{35}S-cysteine (Dupont Canada; >600 Ci/mmol). The dishes were incubated for 3 more hours at 37°C in 5% CO_2. The supernatant was saved for determination of bioactive TNFα, and the adherent cells washed 2-4 times with warm PBS. In a 4°C cold room, the remaining adherent cells were lysed with 300 μL of ice-cold extraction buffer [0.02M Tris HCl, pH 8.0; 0.001M Na_2HPO_4; 0.137M NaCl; 0.5% NP-40] containing 1 mM phenylmethylsulfonyl fluoride and 0.01% globulin-free bovine serum albumin (BSA; Sigma). The lysate was collected into Eppendorf tubes, centrifuged for 1 minute at 10,000 x g and 40 μL of *Staphlococcal aureus*-protein A-Sepharose beads (SAS; Sigma), preblocked by suspension in PBS containing 1% (vol/vol) globulin-free BSA, was added. The mixture was gently rotated for 2 hours at 4°C. The samples were then centrifuged at 10,000 x g and the supernatant transferred to new Eppendorf tubes. 20 μL of unpurified rabbit anti-mouse TNFα (final dilution 1 in 3750) was added and the samples gently rotated at 4°C for 2 hours. 40 μL of preblocked SAS was added and the sample gently rotated for 1 hour. The sample was centrifuged at 10,000 x g, the supernatant discarded and the pellet washed 2 times with ice-cold wash buffer [0.1M Tris HCl, pH 8.0; 0.5M LiCl; 0.5% NP-40] containing 0.1% BSA and 3 times using ice-cold wash buffer without BSA. The tube was drained and 8 to 10 μL of sample buffer containing 2-mercaptoethanol added and the sample heated at 100°C on a heat block for 5 minutes. After immunoprecipitation, samples were kept frozen and, prior to electrophoresis, centrifuged at 10,000 x g for 5 minutes. Electrophoresis was performed with PhastSystem (Pharmacia, Uppsala, Sweden), using 0.5 μL of sample on prepackaged homogeneous sodium dodecylsulfate (SDS)-polyacrylamide gels (Pharmacia) containing 12.5% acrylamide. After electrophoresis, the gels were fixed for 15 minutes in 7% acetic acid followed by a 30 minute soak in an enhancing agent (AMPLIFY; Amersham Canada, Oakville, ON). The gels were dried at 80°C for 30 minutes in a vacuum oven. Gels were analyzed by autofluorography after exposure to preflashed Kodak XAR 5 film with intensifying screens at -70°C for 10 to 30 days.

Culture supernatants

Culture supernatant for testing of biologically active TNFα was collected in two ways. Macrophages were washed and 1-5 x 10^4 cells/mL in serum-free medium (IMDM) added to wells of a 96-well flat-bottom microtiter plate. S-oligo antisense, nonsense, or PBS control was added to appropriate wells and the plate incubated for 2 hours at 37°C in 5% CO_2. FBS was added to give a final concentration of 2.5-5% (vol/vol), the plates incubated for another 2 hours, then cytokine or control medium added and the plates cultured for an additional 24 to 36 hours. The supernatants were collected, pooled where appropriate, and kept frozen until assayed for TNF activity. Alternatively, supernatants were collected from immunoprecipitation experiments (see above) and used to assay TNF activity.

RESULTS

The ability of 22mer S-oligos made antisense to TNFα mRNA to inhibit production of TNFα from cultures of mouse macrophages was assessed in two manners: (1) inhibition of biologically active TNFα detectable in culture supernatants after stimulation with CSF-1 and LPS and (2) inhibition of accumulation of biosynthetically ^{35}S-labeled, cell-associated TNFα propeptide in cultures after stimulation with CSF-1 or LPS. LPS is a major mediator of TNFα production from macrophages (15). Thus, it was important to control for possible LPS contaminants in cytokine preparations and culture medium. We, therefore, used macrophages derived from C3H/HeJ mice which have a defect in their ability to produce TNFα in response to LPS-stimulation (15). Use of these macrophage populations should allow for an assessment of the effects of S-oligos on TNFα production with minimal interference from LPS contaminants. Therefore, inhibition of TNFα production was examined in four macrophage populations: in bone marrow-derived macrophages (BMM) and a growth factor-dependent cell line (S1) of C3H/HeJ origin (LPS-hyporesponsive) and in BMM of C3H/HeN origin (LPS-responsive) and from the LPS-responsive macrophage cell line RAW 264.7 (RAW). Nonspecific effects of S-oligos on TNFα production were assessed by comparison of the effect of antisense to a scrambled base-matched nonsense counterpart.

The effect of S-oligos on the detection of biologically active TNFα

In 12 independent experiments where antisense and nonsense S-oligo-treated macrophages were compared to control cultures for biologically active TNFα production, 8 showed significant inhibition by antisense treatment (data not shown). The sequence-specific inhibition of TNFα protein production was independent of the concentration of the S-oligos over the range 2.5 μM to 18 μM; however, concentrations less than 10μM were slightly more effective (data not shown). Whether antisense S-oligos inhibited TNFα production was related to the manner in which the cells were stimulated. Figure 1 shows relative TNFα production (in percent) of nonsense-treated cultures to control culture (NS/C) and of antisense-treated to nonsense-treated cultures (AS/NS). The former ratio gives insight into the nonspecific effects of S-oligos and the latter of the effects of the specific antisense sequence. When CSF-1 was used as the stimulus (in 7 experiments), antisense S-oligo inhibited production of biologically active TNFα by 84% relative to the nonsense control ($p<0.01$ by Student's t test).. Within error, nonsense did not alter TNFα production relative to the untreated control. However, when LPS was used as a stimulus (in 2 experiments), the average degree of antisense inhibition was reduced to 17% ($p=$n.s.).

These differing results, depending on the stimulus, correlate with the relative levels of secreted TNFα by each stimulus in the presence of nonsense S-oligo (Table 1). The secretion data for the cytokine CSF-1 was abstracted from results with cells of C3H/HeJ origin to avoid possible stimulation by LPS contaminants in the S-oligo preparations. These data show that LPS stimulation of nonsense sequence S-oligo treated cells releases approximately 2.5-fold more TNFα than do CSF-1-stimulated cells. Thus, antisense S-oligos can specifically inhibit TNFα production from mouse macrophages depending on the activation history. Furthermore, the ability of the antisense sequence to inhibit correlates inversely with the TNFα production rate.

The effect of S-oligos on the biosynthesis of TNFα

TNFα protein is highly regulated post-transcriptionally prior to its release from the cell (16-20). Moreover, the propeptide form of TNFα can be membrane-bound and is thought to be a precursor for the mature, released form(s) of TNFα (18). Thus, not all of the secreted TNFα measured in Figure 1 may result from translational activation of TNFα mRNA, but may, instead, be preformed TNFα released during the culture from preexisting propeptide. Thus, the inhibiting effects of antisense S-oligos may be due to negative effects on the release of TNFα rather than on the translation of the protein. Therefore, we used specific immunoprecipitation of biosynthetically ^{35}S-labeled TNFα propeptides to further examine the inhibitory effects of antisense S-oligos at the translational level.

Cell-associated TNFα propeptides immunoprecipitate as multiple isoforms having approximate molecular masses of 24-26 kD, 27-29 kD and 32-36 kD (16, 17). Using RAW 264.7 macrophages, there was no apparent inhibition by antisense S-oligos of propeptide biosynthesis after stimulation with LPS (Figure 2). Three isoforms of TNFα were identified having molecular weights of approximately 24kD, 27kD and 33kD. These isoforms were confirmed to be TNFα using competition experiments with nonradioactive recombinant mouse TNFα (data not shown, see Chapter V). In contrast to RAW cells, when using LPS-stimulated C3H/HeJ-derived S1 macrophages, antisense S-oligo caused a significant reduction in the propetide isoforms produced compared to medium or nonsense controls (Figure 2). Thus, TNFα propeptides were inhibitable in a gene-specific manner by antisense to TNFα, indicating a direct inhibitory effect on the translation of TNFα.

Relative TNFα production by LPS-stimulated mouse macrophage

The macrophage is the major source of TNFα production, particularly after stimulation of these cells with LPS (21, 22). We found that LPS-responsive macrophages (from C3H/HeN mice) produced 1.6 - 10-fold more TNFα after GM-CSF or CSF-1-stimulation, respectively, than LPS-hypo-responsive macrophages (from C3H/HeJ mice) (see Chapter V). Indeed, the ability of the antisense sequence to inhibit TNFα correlates inversely with the TNFα production rate. As seen in Figure 3, LPS stimulates high levels (> 20 ng/mL) of TNFα from RAW 264.7 macrophage but only approximately 10 pg/mL TNFα from the S1 macrophage cell line. Thus, using macrophages which were capable of high TNFα production, such as RAW 264.7, S-oligos were ineffective (Figure

2). In contrast, when using macrophages which produce low levels of TNFα, such as macrophages derived from C3H/HeJ mice, antisense S-oligos were shown to be very effective at blocking TNFα protein production (Figure 2).

DISCUSSION

In this present work, we have used synthetic phosphorothioated analogues of oligodeoxynuleotides to determine the feasibility of using these S-oligo sequences, made antisense to TNFα mRNA, to inhibit endogenously produced TNFα. S-oligos are very resistant to degradation, have a relative long half-life in culture systems, show efficient RNA hybridization and cell delivery (11). Thus, S-oligos should have potential usefulness for *in vitro* studies of the role for endogenously produced TNFα.

We find that antisense S-oligos can inhibit in a sequence-specific manner TNFα protein production at the translational level. The successful inhibition of TNFα protein production appears to depend on the macrophage used. Whereas TNFα production from HeJ-derived macrophages was readily inhibitable, TNFα produced by RAW 264.7 macrophages was not, and production of TNFα from HeN-derived macrophages was only partially inhibited. These differing results, depending on the macrophage, correlate with the relative levels of TNFα capable of being produced by the different macrophage populations.

A role, if any, for TNFα, operative in autocrine or cell-cell interactions has not been well examined. Indeed, the presence of a biologically active membrane-bound propeptide form of TNFα suggests a potential physiologic role in cell-cell communication (18). However, experiments designed to define a role for autocrine or cell-associated TNFα have proceeded slowly. Undoubtedly, this is partly due to the difficulty in inhibiting endogenously produced TNFα protein, either at the translational or protein level. Indeed, RAW 264.7 macrophages have been shown to have a high rate of TNFα transcription and translation following stimulation with LPS, with 1-5% of the total secreted protein being TNFα (21). This makes inhibition of TNFα protein production very difficult. However, the work of Himeno *et al* (23) using transfection of a plasmid construct containing sequences made either antisense or sense to TNFα mRNA has provided some insight into the function of endogenously produced TNFα. Their elegant work indicated that expression of endogenous TNFα, in cells which are normally killed by exogenously added TNFα, protects against the cytolytic effects of TNFα. Furthermore, these experiments showed that it is possible to significantly inhibit TNFα protein production using antisense sequences to TNFα mRNA.

Our combined results using immunoprecipitation and measurement of biologically active TNFα indicates that synthetic 22-mer S-oligo sequences made antisense to TNFα mRNA can effectively and specifically inhibit the translation of TNFα protein from mouse macrophages. The ranking of the effectiveness of antisense S-oligos on various macrophage populations is C3H/HeJ > C3H/HeN > RAW 264.7. This may limit the potential usefulness of antisense S-oligos for inhibiting TNFα protein production in certain macrophage systems. This is the first report of successful blocking of TNFα protein production using synthetic antisense oligodeoxynucleotides.

Table 1

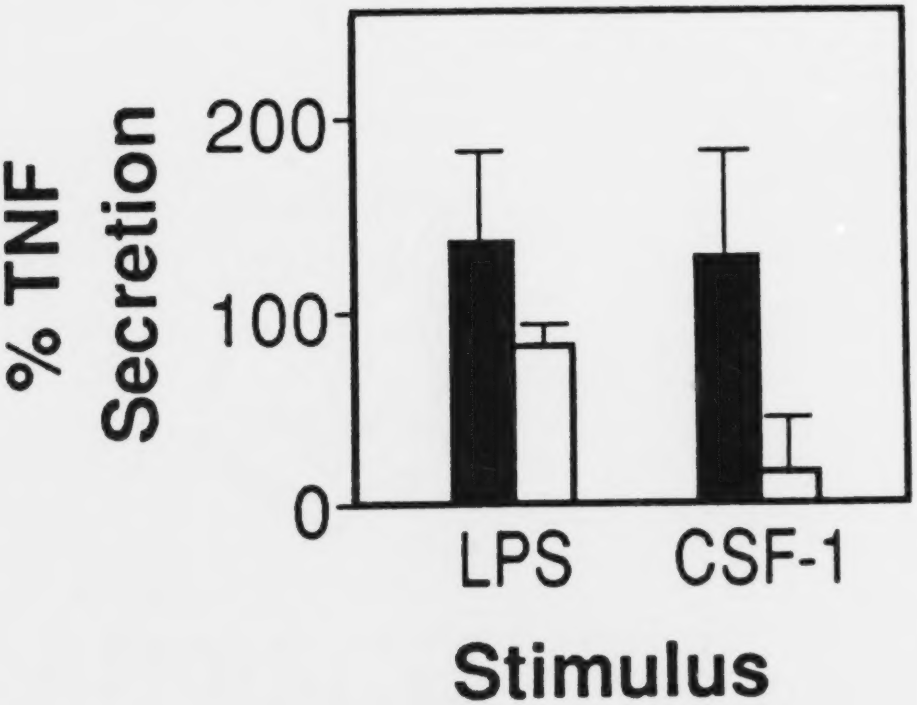
Secretion of biologically active TNFα from macrophages stimulated by CSF-1 and LPS in the presence of nonsense S-oligo sequences*

Stimulus	n	TNFα levels (pg/mL)
CSF-1	3	17 ± 11
LPS	2	40 ± 9

---

* TNFα assayed as described in detail in the Methods. Data for CSF-1 are pooled from experiments carried out on C3H/HeJ BMM and C3H/HeJ-derived S1 cells. Data for LPS comes from pooled results carried out with RAW 264.7 cells and high LPS-stimulated S1 cells.

Figure 1



Alteration of biologically active TNFα production by mouse macrophages as a function of stimulation and treatment with phosphorothioated oligodeoxy-nucleotides (S-oligos). Solid bars: Mean ratio of TNFα production after treatment with base-matched nonsense S-oligo relative to the untreated controls (referred to in the text as NS/C). Open bars: Mean ratio of TNFα production after treatment with antisense S-oligo relative to production after treatment with the nonsense control (referred to in the text as AS/NS). Data for cytokine histograms pooled from 7 separate experiments (7 for CSF-1) carried out with C3H/HeJ and C3H/HeN BMM and S1 cells. Data for LPS histogram pooled from two separate experiments using RAW 264.7 and S1 cells.

Figure 2

Immunoprecipitation analysis of TNFα propeptides produced by the RAW 264.7 and C3H/HeJ-derived S1 macrophage cell lines after treatment with phosphorothioate oligodeoxynucleotides (S-oligos) and stimulation with LPS. Macrophages were incubated, using serum-free conditions, in the presence of medium CONTROL (lanes 1 and 4), 5μM 22-mer S-oligos, made ANTISENSE to TNFα mRNA (lanes 2 and 5), or 5μM 22-mer S-oligo NONSENSE sequence (lanes 3 and 6), base-matched to the antisense S-oligo, for 2 hours, then made 10% (vol/vol) with dialyzed FBS, incubated for 1 hour, then 5μg/mL LPS added, and the proteins labeled with a pool of ^{35}S-methionine and -cysteine for 3 hours. Rabbit antimouse TNFα was used to immunoprecipitate cell lysates and analyzed by electrophoresis using 12.5% SDS-polyacrylamide gels. Autofluorography was done using preflashed Kodak XAR 5 film with intensifying screens at -70°C for 30 days. The position of molecular weight markers are shown on the right, and TNFα immunoreactive bands are indicated by their apparent molecular weights at the left.

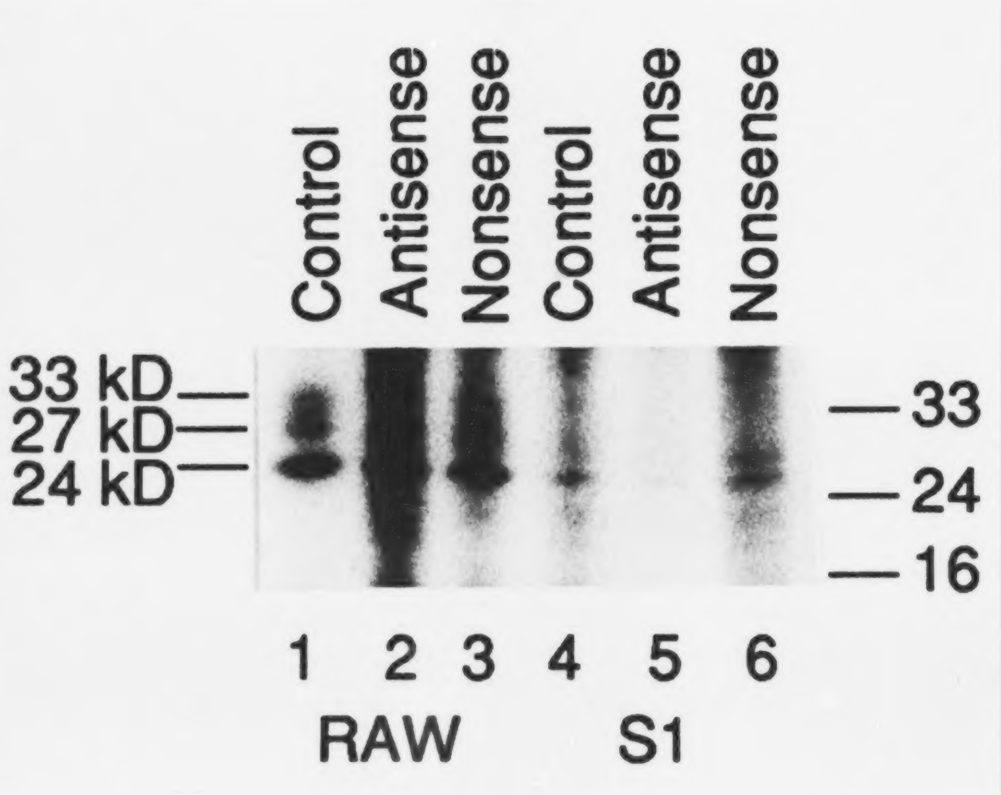
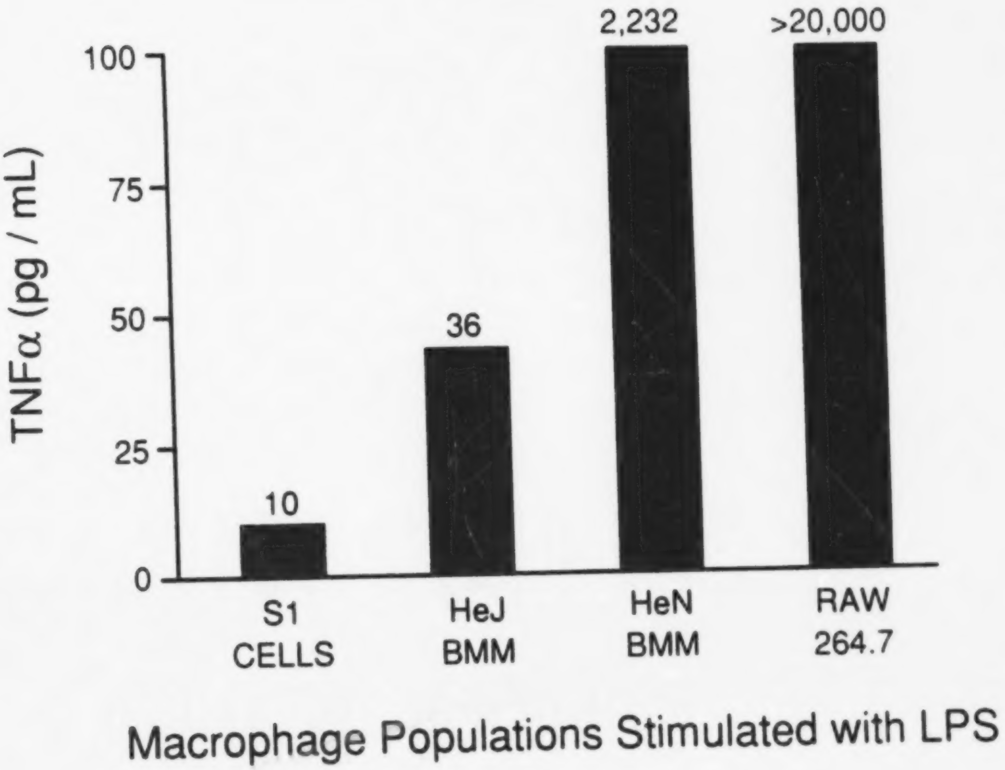

Control
Antisense
Nonsense
Control
Antisense
Nonsense
33 kD
27 kD
24 kD
33
24
16
1
2
3
4
5
6
RAW
S1

Figure 3

Production of TNFα from macrophages stimulated with LPS



TNFα production from C3H/HeJ-derived (S1 cells, HeJ BMM), C3H/HeN (HeN BMM) and RAW 264.7 macrophages after stimulation with LPS. Total TNFα (lysate + culture supernatant) was measured after 4 hours of culture in the presence of 5 μg/mL LPS. TNFα was quantified using the L929-8 bioassay (12) by comparison to a pure, recombinant mouse TNFα standard.

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APPENDIX 3

NONSPECIFIC INHIBITION OF MOUSE MACROPHAGE PROLIFERATION INDUCED BY PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

INTRODUCTION

Numerous reports have emerged over the past few years indicating that synthetic antisense oligodeoxynucleotides are effective, gene-specific, down-modulators of protein translation (Stein and Cohen, 1988). Many variations on the phosphodiester backbone of these synthetic molecules have been designed to enhance the ideal properties required for the effective use, both *in vitro* and *in vivo*, of synthetic antisense sequences. Thus, for greatly increased stability in the presence of nucleases, sulfur analogues of oligodeoxynucleotides were developed. These phosphorothioate analogues have since been shown to combine water solubility, effective hybridization for duplex formation, cellular uptake via an 80 kD receptor, and susceptibility to RNase-H cleavage, along with significant nuclease-resistance, thus, making sulfurized-oligodeoxynucleotides (S-oligos) potentially superior to unmodified oligos, or other types of modified oligos, i.e. α-oligos or methylphosphonate (Stein *et al.*, 1988; Stein and Cohen, 1989; Cazenzve *et al.*, 1989; Campbell *et al.*, 1990).

S-oligos have been successfully used to inhibit replication of a number of human viruses, including human immunodeficiency virus (Matsukura *et al.*, 1987; Agrawal *et al.*, 1989), herpes simplex virus type 2 (Gao *et al.*, 1989) and influenza virus (Leiter *et al.*, 1990). In addition, S-oligos have been shown to inhibit the expression of mRNA for the proto-oncogenes c-*myc* (Loke *et al.*, 1988), bcl2 (Reed *et al.*, 1990) and the transferrin receptor (Ho *et al.*, 1990) and IL-1β (Manson *et al.*, 1990) proteins.

Although S-oligos possess properties which make them highly desirable for use *in vitro* and *in vivo*, and successful inhibition of certain gene products has been achieved, these modified oligodeoxynucleotides have not yet been fully investigated for their gene-specific or -nonspecific effects. Indeed, S-oligos are known to interact with and bind proteins (Eckstein, 1986), have been shown to result in gene-nonspecific inhibition of protein production (Manson *et al.*, 1990), which may be concentration and length-dependent (Cazenave *et al.*, 1989; Stein and Cohen, 1989), and also to bind to reverse transcriptases (Majumdar *et al.*, 1989) and DNA polymerases (Gao *et al.*, 1989).

The above mentioned findings suggest potential problems associated with the use of S-oligos. This, coupled with the limited amount of work thus far generated using S-oligo antisense sequences to specifically inhibit mammalian protein production, strongly indicates that more information regarding the gene-specific effects of S-oligos is needed before the efficacy of these modified oligodeoxynucleotides can be estimated.

Previously, we have shown that the macrophage product tumor necrosis factor (TNF)α can synergistically cooperate with the macrophage growth factor CSF-1 to increase the proliferation of homogeneous populations of both primary mouse macrophages and a macrophage cell line (Branch *et al.*, 1989). Thus, endogenous production of TNFα by macrophages may provide an autocrine stimulatory loop (Browder *et al.*, 1989). Indeed, our results using neutralizing anti-TNF indicated that the CSF-1-mediated proliferative response of a primary mouse macrophage population could be partially inhibited (see Chapter V). This slight, but significant ($p<0.05$) effect was thought to reflect the inability of the experimental system to effectively limit the internal synthesis of TNFα. Thus, the study addressed only an external autocrine growth loop. More recently, we used 22-mer S-oligos made antisense to TNFα mRNA and a 22-mer scrambled, base-matched S-oligo nonsense sequence to show that sequence-specific inhibition of TNFα protein could be achieved using antisense S-oligo to TNFα mRNA (see Appendix 2). We planned to use this technique to address the question of the role of endogenously produced TNFα in the proliferative response of mouse macrophages to mitogenic stimulation by macrophage growth factors. We here report the results of that study.

We find that S-oligos inhibit, in an apparent nonspecific manner, proliferation of mouse macrophages. Furthermore, we find that this inhibition is not a toxic effect, but may be related to the length and/or sulfurization of the phosphorothioate oligodeoxynucleotides.

MATERIALS AND METHODS

Cells

Primary bone marrow-derived macrophages (BMM) were prepared from the femurs of C3H/HeJ or C3H/HeN mice as previously described (Branch and Guilbert, 1986). Adherent cells at day 7 of culture (d7 BMM) were harvested and used in proliferation and immunoprecipitation assays. The procedure yields a population of growth factor-dependent macrophages of established homogeneity (Branch *et al.*, 1989). The C3H/HeJ-derived v-*myc*-transformed, growth factor-dependent macrophage cell line S1 (Branch *et al.*, 1989), was maintained in culture on plastic Petri dishes in Iscoves's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) supplemented with 15% (vol/vol) fetal bovine serum (FBS, flow Laboratories, McLean, VA), 20% (vol/vol) L-cell conditioned medium and GM-CSF (5 ng/mL). Mouse L929-8 fibroblastoid cells were isolated from parent L929 cells as previously described (Branch *et al.*, in press) and maintained in tissue culture flasks with IMDM supplemented with 10% (vol/vol) FBS.

Cytokines and antibodies

Pure, recombinant human CSF-1 (2.1×10^6 U/mL) was a gift of Genetics Institute, Boston, MA. Pure recombinant mouse GM-CSF was from Immunex Corp., Seattle, WA, or from GLAXO, Mississauga, ON. Pure, recombinant mouse TNFα (1.2×10^7 U/mg) and neutralizing rabbit anti-mouse TNF, was obtained from Genentech, South San Francisco, CA. Pure, recombinant human TNFα was obtained from Hoffmann-La Roche, Basel, Switzerland.

Synthetic phosphorothioate oligodeoxynucleotides

Phosphorothioate oligodeoxynucleotides (S-oligos) were chosen for their effective hybridization for duplex formation (Stein *et al.*, 1988), significant nuclease-resistance (Stein *et al.*, 1988; Campbell *et al.*, 1990), solubility (Stein and Cohen, 1988), and specific receptor-mediated cellular uptake (Loke *et al.*, 1989). Based on the published cDNA sequence for mouse TNFα (Pennica *et al.*, 1985), an S-oligo having the sequence 5'-GGATCATGCTTTCTGTGCTCAT-3', which was complementary to the first 22 nucleotides of the sense strand of mouse TNFα mRNA, starting with nucleotide 144 of the translation initiation start site codon (AUG), was purchased from Synthecell Corporation, Rockville, MD. The FASTA system gene-banks search (Pearson and Lipman, 1988) indicated that this nucleotide sequence, when analyzed in the antisense direction, had a high degree of homology with various animal TNFα genes without significant homologies to other known genes, when evaluated in either the sense or antisense direction. A number of base-matched 22-mer S-oligo nonsense sequences were evaluated, both in the sense and antisense direction, for significant homologies with other known genes using FASTA (Pearson and Lipman, 1988). The nonsense sequence 5'-GTCAGGTCGTTACTCGTTTACT-3' was purchased from Synthecell. This sequence was base-matched to the antisense sequence and designed to have minimal homologies to any known genes. Also purchased from Synthecell was a 22-mer S-oligo sequence (5'-TGCCTATGTCTCAGCCTC TTCT-3'), made sense to the TNFα mRNA, starting at nucleotide 231 of the cDNA for TNFα. This sense sequence was selected to contain the same number of thymidine nucleotides as the antisense sequence. In addition, 10-mer S-oligo antisense (5'-CTGTGCTCAT-3') and nonsense (5'-CGTTACTCGT-3') sequences were purchased from the Regional DNA Synthesis Laboratory, Calgary, Alberta. Also, 15-mer S-oligos that were made antisense to the proto-oncogenes c-*myc* (5'-CACGTTGAGGGGCAT-3') and c-*fos* (5'-ACCCGAG AACATCAT-3') were purchased from the Regional DNA Synthesis Laboratory. S-oligos were diluted in phosphate buffered saline (PBS) and kept at -70°C until used.

Proliferation assays and culture supernatant

The assay system for studies of macrophage proliferation in the presence of synthetic oligodeoxynucleotides is based on a previous report (Birchenall-Roberts *et al.*, 1990). Day 7 BMM or S1 macrophages were harvested from Petri dishes following 5 minutes of treatment at 4°C with sterilized 5 mM ethylenediamine tetraacetic acid (EDTA) in PBS, and washed four times with sterile PBS (Branch and Guilbert, 1986). One, 5 or 20 thousand cells in 40 μL of serum-free IMDM were added to wells of a 96-well flat-bottom microtiter plate, 10 μL of S-oligo or diluent control (PBS) immediately added to appropriate wells, and the plate incubated for 2 hours at 37°C in an atmosphere of 5% CO_2 in air. Ten μL FBS was added to give a final concentration in the wells of 2-5% (vol/vol). After an additional 2 hours of incubation, cytokines, or control medium, were added to appropriate wells and the plate cultured for 24 to 60 hours. Proliferation was quantitated by addition of ^{3}H-TdR (1 μCi/microwell; New England Nuclear) for the last 4 hours of culture, the cells harvested onto glass-fiber paper and ^{3}H-TdR incorporation measured on a scintillation counter (Branch and Guilbert, 1986). Alternatively, the total viable cell number was determined colorimetrically, using the dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] abbreviated MTT (Mosmann, 1983; Branch and Guilbert, 1986). After culture, the wells are decanted and 50 μL of a 10% MTT solution added to all wells. The plates are incubated at 37°C for 4 hours, 0.04N HCl-isopropanol solution added and all wells mixed thoroughly. The plates are read on a Dynatech MR600 Microplate reader with the test wavelength of 570 nm and a reference wavelength of 630 nm (Branch and Guilbert, 1986).

DNA analysis

Supernatant was completely removed from macrophages cultured on 96-well microtiter plates, 50μL of 0.1 M NaOH was added to each well and the plate incubated at 60°C for 15 minutes or allowed to sit covered at room temperature overnight. Fifty μL of HCl/Tris buffer (a 1:1 mixture of 0.1M Tris, pH 8.0 and 0.2M HCl) was added to each well to bring the pH to neutrality. In a cuvette, 100 μL of solubilized and neutralized sample, or calf thymus DNA standard, was mixed with 1.9 mL of dye buffer [10 mM Tris, 1 mM EDTA, 0.1 M NaCl, pH 7.4, containing Hoechst 33258 dye (Sigma Chemical) at 100 ng/mL]. Output fluorescence was read out directly as ng DNA on a Perkin-Elmer LS5 DNA fluorometer (Perkin-Elmer, Palo Alto, CA) set at an excitation wavelength of 365 nm, and an emission wavelength of 458 nm.

[^{3}H] Tritiated leucine uptake

The relative protein synthetic capacity of cells cultured with and without cytokines and S-oligos was evaluated in terms of uptake of TCA precipitate tritiated leucine into cellular compartments. Macrophages cultured as described above in 100μL microwells were pulsed for 4 hours at 37δ C with 1 μCi L-[4,5-^{3}H] leucine (4.4 - 7.0 TBq/mmol, Amersham, Oakville, Ontario), then the medium removed, the cells washed twice with warm PBS and lysed in 50 μL 0.5% SDS, 0.05M Tris-HCl, pH 8. Protein in the lysate was immediately precipitated by addition of an equal volume of 40% (w/v) trichloroacetic acid (TCA), then harvested onto glass fiber filters that had been pre-soaked in 10 mM DL-leucine (Sigma, St. Louis, Mo) and washed with 5% TCA. After two washes with 5% TCA the tritium content of the filters was determined by scintillation counting as described above for ^{3}H-TdR uptake proliferation studies.

RESULTS AND DISCUSSION

Effect of S-oligos on macrophage proliferation

It has been hypothesized that endogenous production of TNFα by mouse macrophages may be essential for the CSF-1-induced mitogenic stimulation of these cells (Branch *et al.*, 1989; see Chapter V). We, therefore, used S-oligos made antisense to TNFα mRNA to determine their effect on the proliferation of mouse macrophages. Using 10μM S-oligos we found strong inhibition of ^{3}H-TdR incorporation when either antisense or scrambled, base-matched nonsense sequences were used (Figure 1). Reasoning that this apparent nonspecific effect may be related to the concentration of th S-oligos, we next determined the dose-response for this inhibitory effect.

Using ^{3}H-TdR incorporation as an indicator of macrophage proliferation, we found S-oligos to have a dose-dependent inhibitory effect on macrophage proliferation over the concentration range 0.6μM to 20 μM (Figure 2**A** and **B**). Furthermore, we were unable to distinguish a difference in the effects of the antisense TNFα S-oligo from either the base-matched nonsense control (or a thymidine-matched sense control [Figure 3]) on the proliferation of mouse macrophages. Thus, despite sequence-specific inhibition of TNFα protein (see Appendix 2), no clear "window" demonstrating a sequence-specific effect on macrophage proliferation was found.

Further studies using reduction of the dye MTT, used for the determination of total viable cell content (Mosmann, 1983), showed a much less dramatic, although similar, dose-inhibitory response when using the S1 macrophage cell line (Figure 2**c**). However, S-oligos appeared to have little effect on the total viable cell content when using d7 HeJ BMM (Figure 2**d**), although these cells were severely inhibited in their incorporation of ^{3}H-TdR over the same concentration range (Figure 2**b**). These findings suggest that the inhibitory results using uptake of ^{3}H-TdR may partly result from competition for the radiolabeled thymidine by the nonradioactive thymidine nucleosides released after degradation of the S-oligo sequences. As further support for this, macrophage proliferation stimulated by CSF-1 was examined using both ^{3}H-TdR incorporation and reduction of MTT in the presence or absence of 40μM antisense S-oligo sequences (Figure 4). Although by ^{3}H-TdR uptake it appears that antisense S-oligo can inhibit CSF-1-stimulated macrophage proliferation of the S1 cell line and primary BMM (Figure 4B), no clear differences in the effect of S-oligos antisense, compared to the CSF-1 control, on the total viable cell number as measured using MTT reduction was evident (Figure 4a) with the exception of the S1 cell line. Thus, these results are supportive of thymidine competition for the observed inhibitory effects. However, a lack of an inhibitory effect of S-oligo antisense when using RAW 264.7 macrophages (Figure 4) and inhibition of S1 cell growth as measured by either ^{3}H-TdR or MTT, argues that cold competition for ^{3}H-TdR may not be the whole story for the observed inhibitory effects of S-oligos.

Additional experiments where human and mouse TNFα was added to the CSF-1-stimulated cultures of macrophages containing 22-mer S-oligo could partially reverse the inhibitory effect of S-oligos (Figure 5), also argues that cold competition of the ^{3}H-TdR by S-oligos does not adequately explain the apparent inhibitory effect.

A possible explanation for the differences seen when using ^{3}H-TdR uptake compared with MTT reduction may involve cell-cycle times. If very few BMM are proliferating, one would predict only a small increase in MTT reduction, while ^{3}H-TdR uptake may still be relatively high. We know that S1 cells have a doubling time of 20 hours, therefore, we would expect the MTT result to approximately double over this time period. We also know that BMM have a decreased rate of growth compared to S1 cells. Thus, an alternative explanation is that S-oligos inhibits proliferation of S1 and BMM without killing the cells so that one doesn't see a dramatic decrease in MTT.

Further examination as to the reason for this paninhibitory effect of S-oligos showed that it was not due to a cell toxicity effect as trypan blue exclusion studies indicated that >98% of cells remain viable. Furthermore, using ^{3}H-leucine incorporation and DNA measurements indicated that total protein or DNA synthesis was not significantly affected in the presence of S-oligos compared to control cultures (Table 1).

To examine whether the length of the S-oligos (Stein and Cohen, 1989) may be partly responsible for the inhibitory effect obtained when using ^{3}H-TdR uptake, we compared the effects of the 22-mer sequences to related 10-mer and unrelated 15-mer S-oligo sequences (Table 2). The 10-mer S-oligo sequences appeared to have little inhibitory effect on the growth of CSF-1-stimulated macrophages up to concentrations of 25 μM, while 15-mer sequences (4 μM) resulted in slight to moderate inhibition (4% to 50%). In contrast, 22-mer S-oligos inhibited the CSF-1 response by 50% to 80% at 2.5 μM concentrations. Since the thymidine content of the 10-mer and 15-mer sequences are the same and approximately half of the 22-mer sequences, and since the concentrations of the 10-mer and 15-mer sequences were adjusted so that levels of thymidine approached those of the 22-mer sequences, these results suggest that the nonspecific inhibitory effects of S-oligos on macrophage growth may be, at least, partly due to the length of the sequences.

The role of the sulfurs (possibly as sulfhydryl groups), is unclear, although adjusting the concentrations of 10-mer, 15-mer and 22-mer S-oligo sequences, in an attempt to compensate for variability in sulfur content suggests that the phosphorothioate groups are not involved in the inhibition effect on macrophage growth (Table 2).

Thus, further investigation of the mechanism of these nonspecific effects and their resolution is warranted before S-oligos can be used to address questions regarding function of endogenously produced TNFα on the proliferation of mouse macrophages.

Table 1

Effect of S-oligo sequences on protein and DNA synthesis

HeJ BMM stimulated with:	^{3}H-leucine uptake	DNA
Medium alone	319 ± 14	199 ± 14
GM-CSF	1,267 ± 243	238 ± 15
CSF-1	1,538 ± 251	361 ± 29
GM-CSF + AS	1,072 ± 135	243 ± 13
GM-CSF + NS	1,031 ± 123	238 ± 27
CSF-1 + AS	1,538 ± 371	369 ± 39
CSF-1 + NS	1,560 ± 413	328 ± 37

- S-oligos were used at a final concentration of 2.5μM.
- C3H/HeJ BMM were cultured at 10^4 cells per 100 μL microwell for 24 hours in medium alone, GM-CSF (50 ng/mL) or CSF-1 (50 U/mL) with or without S-oligos.
- ^{3}H-leucine uptake determined as described in Materials and Methods

AS antisense S-oligo to TNFα mRNA

NS scrambled, base-matched nonsense S-oligo control sequence (see Materials and Methods)

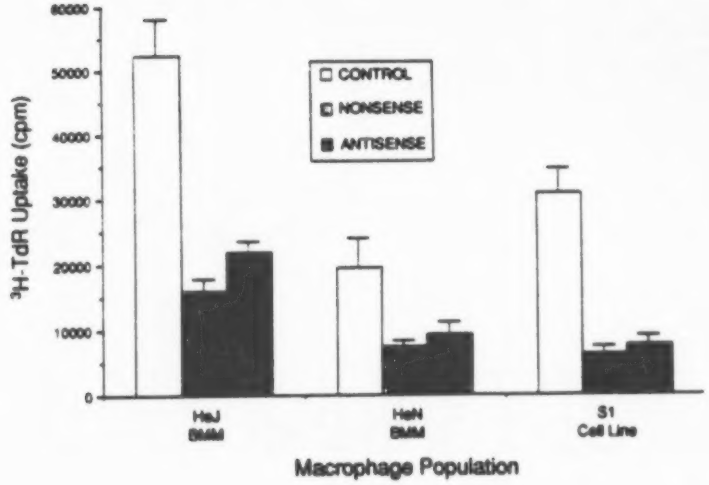
Table 2
Effect of S-oligo length on the nonspecific inhibition of the mitogenic stimulation of mouse macrophage proliferation

				Percent Inhibition of CSF-1 Response using:		
S-oligo[+]	Length	Target mRNA	[S-oligo]	S1 Cell Line	HeJ BMM	HeN BMM
AS	10mer	TNF	25 μM	0	0	0
NS	10mer	TNF	25 μM	0	0	0
AS	15mer	c-*myc*	4 μM	54	38	18
AS	15mer	c-*fos*	4 μM	33	29	4
AS	22mer	TNF	2.5 μM	75	58	52
NS	22mer		2.5 μM	80	70	62
S	22mer	TNF	2.5 μM	81	73	62

[+] AS	antisense sequence
NS	nonsense sequence
S	sense sequence

Proliferation assays were carried out as described in the Materials and Methods.

Figure 1



Proliferation studies using S-oligos made **antisense** for TNFα mRNA and a scrambled, base-matched **nonsense** sequence control. Macrophage populations (5 x 10^4 cells/mL) were cultured in serum-free medium, in the presence of 2.5μM S-oligos, or **control** medium, for 2 hours, the culture made 2.5% (vol/vol) with FBS, the cells cultured an additional 2 hours, 7 ng/mL CSF-1 added, and then cultured for 36 hours. Results are represented as the ^{3}H-TdR uptake (cpm) ± standard deviation of quadruplicate determinations.

Figure 2

The dose-inhibitory response of S-oligos on CSF-1-stimulated macrophage proliferation. Macrophage populations (5 x 10^4 cells/mL) were cultured in serum-free medium, in the presence of varying concentrations of S-oligos made antisense to TNFα (solid boxes) or a nonsense sequence (open boxes), for 2 hours, the culture made 2.5% (vol/vol) with FBS, the cells cultured an additional 2 hours, 70 ng/mL CSF-1 added, and then cultured for 24 hours. Results are represented as the ^{3}H-TdR uptake (cpm) ± standard deviation (SD) of triplicate assays for S1 cells (panel A) or d7 HeJ BMM (panel B), or the optical density ($OD_{570-630}$ ± SD of triplicate assays, using MTT reduction, for S1 cells (panel C) or d7HeJ BMM (panel D). The striped box represents the mean ± SD of CSF-1 alone.

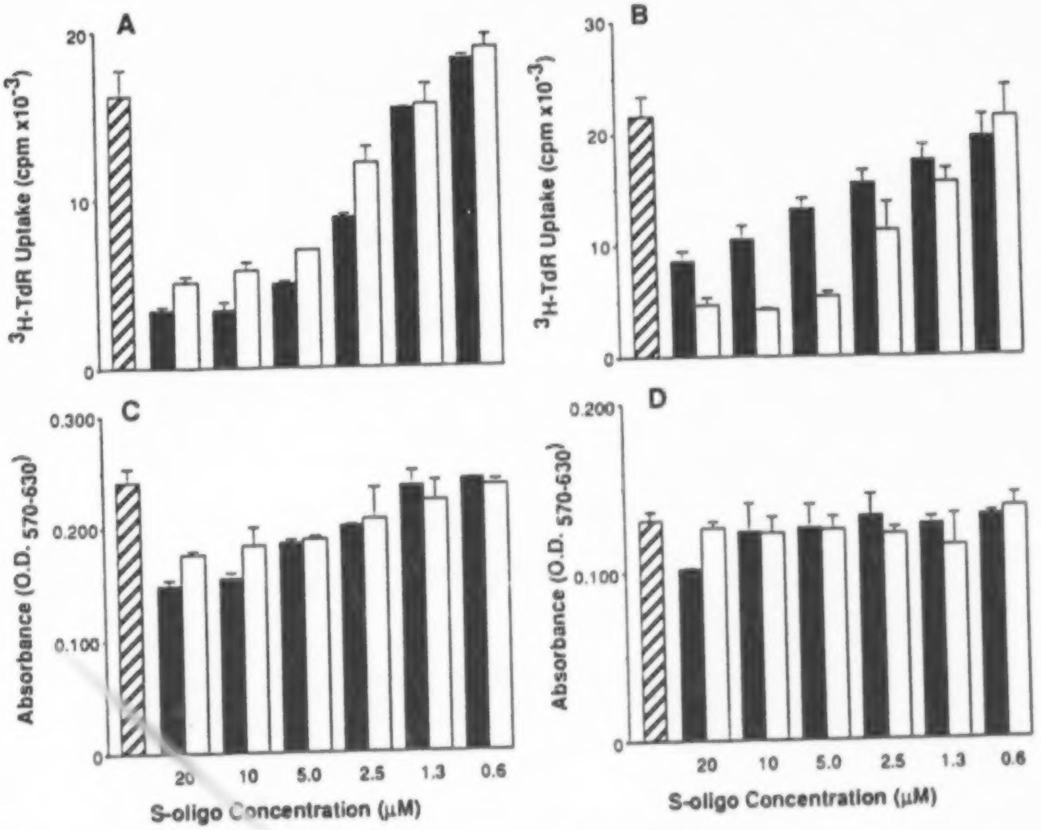
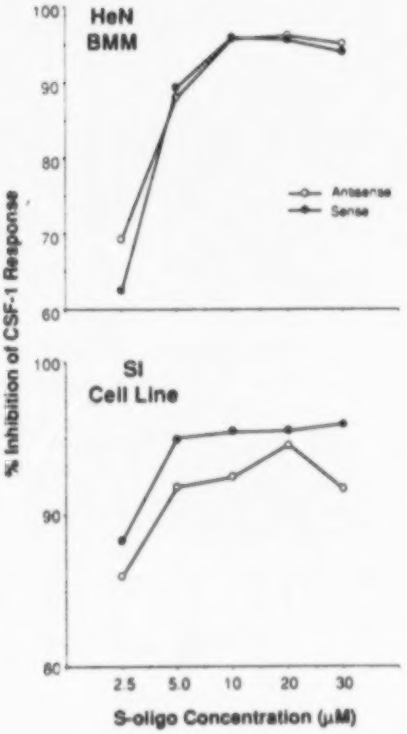

A
B
C
D
^{3}H-TdR Uptake (cpm x10^{-3})
0
10
20
30
Absorbance (O.D. 570-630)
0
0.100
0.200
0.300
20
10
5.0
2.5
1.3
0.6
S-oligo Concentration (μM)

Figure 3



The dose-inhibitory response of S-oligos on the CSF-1-stimulated proliferation of primary d7 HeN BMM and the S1 macrophage cell line. 5×10^4 cells/mL were cultured with CSF-1 alone (7 ng/mL) or CSF-1 plus 22-mer S-oligos made antisense (○) or sense (●) to TNFα mRNA, for 36 hours. The results are presented as the percent inhibition of the ^{3}H-TdR uptake using CSF-1 alone compared to CSF-1 in the presence of S-oligo sequences. Each point represents the mean of quadruplicate determinations.

Figure 4

The effect of S-oligo sequences made antisense to TNFα mRNA on the CSF-1-stimulated growth of mouse macrophages. 10^5 cells/mL were cultured with **control** CSF-1 alone (100 ng/mL: solid boxes) or CSF-1 plus 22-mer S-OLIGO **antisense** (40 μM; open boxes) for 24 hours. **A.** The total viable cell content determined by reduction of MTT, represented as the absorbance (OD x 10^3) ± standard deviation of triplicate determinations. **B.** The ^{3}H-TdR incorporation ± standard deviation of triplicate determinations.

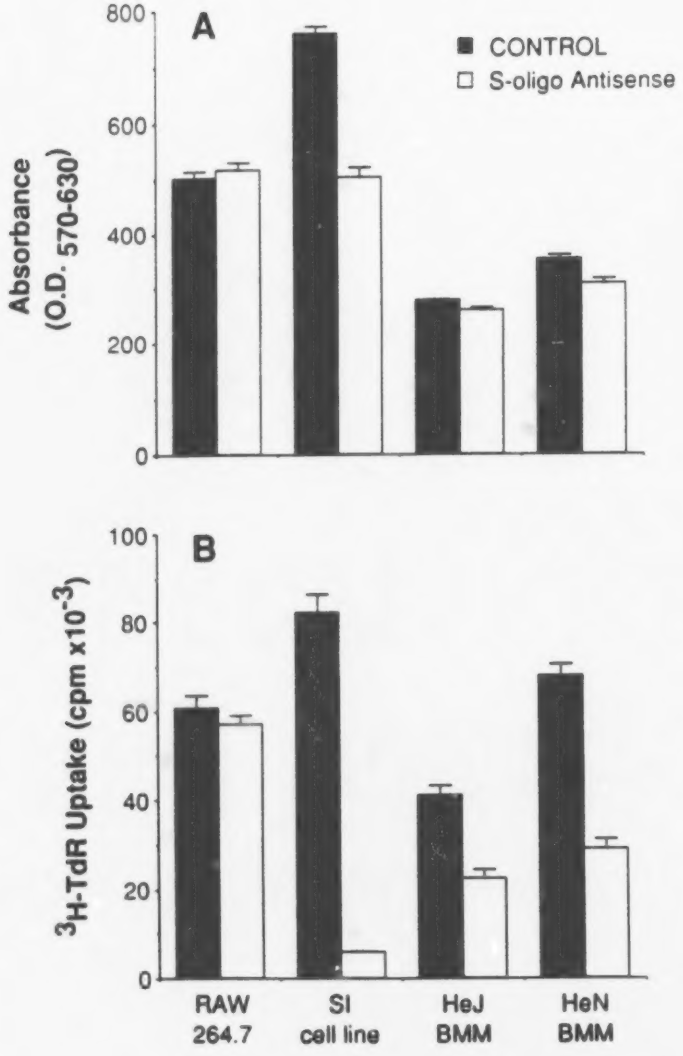
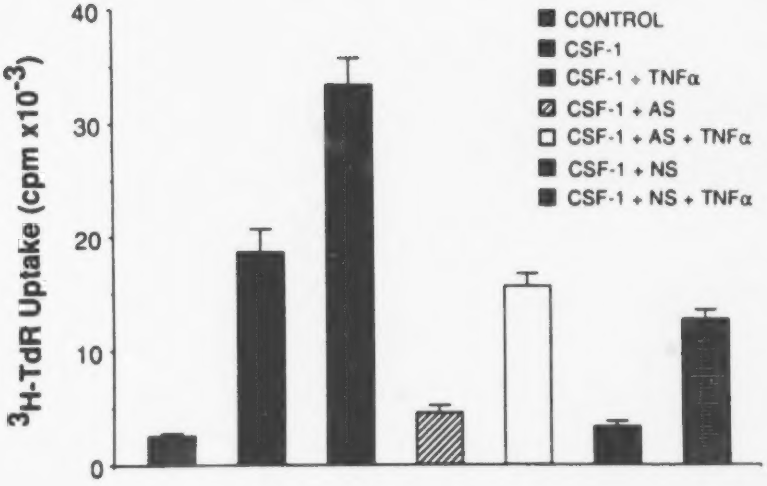

A
CONTROL
S-oligo Antisense
Absorbance (O.D. 570-630)
0
200
400
600
800
B
3H-TdR Uptake (cpm x10-3)
0
20
40
60
80
100
RAW 264.7
SI cell line
HeJ BMM
HeN BMM

Figure 5



The effect of S-oligos on CSF-1 and TNFα-stimulated proliferation of the S1 macrophage cell line. 5 x 10^4 cells/mL were cultured with CSF-1 (100 ng/mL), recombinant mouse TNFα (100 ng/mL), with or without 5 μM 22-mer S-oligos made antisense (AS) or nonsense (NS) to TNFα mRNA. Results are presented as the ^{3}H-TdR incorporation ± standard deviation of quadruplicate determinations.

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APPENDIX 4

PUBLICATIONS WHOLLY OR PARTIALLY BASED ON THIS THESIS:

1. ***Branch DR*** and Guilbert LJ. (1986) Practical *in vitro* assay systems for the measurement of hematopoietic growth factors. Journal of Tissue Culture Methods 10:101-108.

2. ***Branch DR***, Turc J-M and Guilbert LJ. (1987) Identification of an erythropoietin-sensitive cell line. Blood 69:1782-1785.

3. Guilbert LJ and ***Branch DR***. (1989) Regulation of hematopoiesis by growth factors. Proliferation of the murine macrophage as a model for stimulatory and inhibitory effects. In: *Immune Consequences of Trauma, Shock and Sepsis* (E Faist, J Ninnemann and DR Green, eds) Berlin: Springer-Verlag. pp 35-44.

4. ***Branch DR***, Turner AR and Guilbert LJ. (1989) Synergistic stimulation of macrophage proliferation by the monokines tumor necrosis factor-alpha and colony-stimulating factor 1. Blood 73:307-311.

5. Wegmann TG, Athanassakis I, Guilbert LJ, ***Branch D***, Dy M, Menu E and Chaouat G. (1989) The role of M-CSF and GM-CSF in fostering placental growth, fetal growth and fetal survival. Transplantation Proceedings 21:566-568.

6. Guilbert LJ, Athanassakis I, ***Branch DR***, Christopherson R, Crainie M, Garcia-Lloret M, Mogil RJ, Morrish D, Ramsoondar J, Vassiliadis S and Wegmann TG. (1991) The placenta as an immune-endocrine interface: Placental cells as targets for lymphohematopoietic cytokine stimulation. In: *Molecular and Cellular Immunobiology of the Maternal Fetal Interface* (TG Wegmann, TF Gill and E Nisbet-Brown, eds) New York: Oxford University Press, pp 261-276, 1991.

7. ***Branch DR***, Shah A and Guilbert LJ (1991) A specific and reliable bioassay for the detection of femtomolar levels of human and murine tumor necrosis factors. Journal of Immunological Methods 143:251-261.

8. ***Branch DR*** and Guilbert LJ. Macrophage colony-stimulating factor (CSF-1)-stimulated production of tumor necrosis factor-α by growth-competent mouse macrophages. Submitted to Journal of Immunology.